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Functional analysis of MiRNAs involved in the development of legume seeds

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DEDICATORIA

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RESUMO

Para alimentar a população mundial que continua em crescimento, tem de se encontrar novas estratégias que melhorem as técnicas usadas na agricultura. Nos continentes Sul-americano, Asiático e Africano, o uso de leguminosas é prevalente, nomeadamente a soja (*Glycine max*) e o feijão (*Phaseolus vulgaris*). Para melhorar o desempenho destas culturas, é necessário ter um melhor entendimento de como as sementes se desenvolvem e neste trabalho o foco será determinar o efeito da expressão do microRNA (miRNA) miR408 em diferentes estados de desenvolvimento das sementes de *Medicago truncatula* (*M. truncatula*), bem como criar novas plantas transformantes.

Em *P. vulgaris* foram identificados vários miRNAs que actuam no desenvolvimento da semente, nomeadamente o pvu-miR399a e pvu-miR166a. Em *M. truncatula,* já existe informação de que miR408 influencia o número de sementes por vagem. Como ponto de partida, uma análise foi feita para determinar os equivalentes de miR399a e miR166a de *P. vulgaris* e os seus potenciais alvos em *M. truncatula* assim como os potenciais alvos de miR408 de *M. truncatula.* Os resultados desta análise mostraram que os alvos previstos dos três miRNAs estão de acordo com a bibliografia. Usando uma planta previamente transformada (Mtr-MIM408) com um mimic de miR408 (MIM408), um transcrito complementar ao miRNA que diminui a expressão desse, os níveis de expressão de miR408 foram medidos. Dado que as amostras usadas para controlo apresentavam ter expressão de MIM408 não foi possível tirar conclusões. Relativamente à transformação de plantas, os fragmentos com MIM166a e MIM399a foram clonados com sucesso, mas a inserção destes num vector binário para transformar *M. truncatula* não foi possível. Finalmente, as plantas transformadas (Mtr-MIM408) mostraram floração tardia e continham menos sementes por vagem, semelhante ao que se encontra na bibliografia.

Em suma, este trabalho contribuirá para futuros estudos relativos ao impacto que estes miRNAs tem no desenvolvimento das sementes.

Palavras chave:

Desenvolvimento da semente, *Medicago truncatula,* miRNA, MIM, transformação

Abstract

To feed an ever-increasing population worldwide, new strategies for improving agriculture must be found. In the continents of South America, Asia and Africa, there is a prevalent usage of legumes, such as the common beans (*Phaseolus vulgaris*) and soy (*Glycine max*). To boost the yield of these crops, we need to better understand the development of seeds and in this study the focus is on the effect of the expression of micro RNAs (miRNAs), specifically miR408, during the development of the seeds in the model legume *Medicago truncatula* (*M. truncatula*), as well as creating new transformed plants.

In *P. vulgaris* several miRNAs have already been identified to influence the development of the seed, such as miR399a and miR166a and in *M. truncatula*, there are reports of miR408 influencing the number of seeds per pod. As a starting point, a bioinformatic analysis was conducted to search for miR399a and miR166a equivalents in *M. truncatula* and their predicted targets as well as the predicted targets of *M. truncatula* miR408. For all the three miRNAs, the predictions aligned with the bibliography. Using a transformed plant (Mtr-MIM408) that had a mimic of miR408 (MIM408), a transcript complementary to the miRNA that downregulates it, the expression level of miR408 were measured. Because the control samples showed expression of MIM408, no comparison between expression of miR408 and its target between plants could be made. Regarding the transformation of plants, the fragments containing the mimic for miR166a and miR399a were successfully synthetized but failed to be inserted in a binary vector used for transformation *M. truncatula*. Finally, the transformed plants showed delayed blooming and had fewer seeds per pod, similar to what is found in literature.

In short, this work will be a steppingstone in the process of understanding the impact of the expression of these miRNAs in seed development.

Seed development*, Medicago truncatula,* miRNA, MIM, transformation

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Abbreviations and Acronyms

µg - microgram

µl - microlitre

µmol - micro mole

AP2 - APETALA2

ARF - Auxin Response Factor

bp - base pairs

CaMV - cauliflower mosaic virus

cDNA - complementary DNA

cPCR - colony polymerase chain reaction

DCL - Dicer-like protein

DNA - deoxyribonucleic acid

g - unit of acceleration

HD-ZIP III - class-III homeodomain-leucine zipper

HYL1 - HYPONASTIC LEAVES1

IPS1 - INDUCED BY PHOSPHATE STARVATION1

LB - Luria broth medium

m - meter

M – molar

MIM - mimic of a specific microRNA

miRNA – microRNA

ml - mililitre

mM - millimolar

mol - mole

mRNA - messenger RNA

MS - Murashige and Skoog media

MYB - MYELOBLASTOSIS

NAC - NO APICAL MERYSTEM

ng - nanogram

nmol - nanomol

nt - nucleotide

PCR - polymerase chain reaction

PHB - PHABULOSA

PHO2 - PHOSPHATE2

PHR1 - MYB TF PHOSPHATE STARVATION RESPONSIVE1

PHV – PHAVULOTA

Pi - inorganic phosphate

PLC - Plantacyanin

phasiRNA - phased small interference RNA

qPCR - quantitative polymerase chain reaction

RAN - GTP-binding nuclear protein Ran

REV - REVOLUTA

RISC - RNA induced silencing complex

RNA - ribonucleic acid

RT - reverse transcription

s – second

SE - SERRATE

siRNA - small interference RNA

S.O.C. - Super Optimal Catabolite

SPL - Squamosa Promoter-Binding Protein-Like

sRNA - small RNA

TBE - Tris-Borate-EDTA

TF - transcription factor

Introduction

Importance of legumes

Legumes, from the *Fabaceae* family, have a key role in feeding the world as the second most important family of plants used for crops, only behind *Gramineae* (Graham & Vance, 2003). Some important species of legumes include the common bean (*Phaseolus vulgaris*), soybean (*Glycine max*), pea (*Pisum sativum*) and cowpea (*Vigna unguiculata*). Unlike the Gramineae, such as rice and wheat, that have a low content in proteins, legumes such as the soybean and the common bean are a great source of proteins, amino acids and minerals and are especially important in diets of Asia and South America respectively (Graham & Vance, 2003). In addition, most legumes form a symbiosis with bacteria that fixate atmospheric nitrogen forming symbiotic root nodules. Due to these aspects, legume crops are often used to improve soil fertility and can be used as forage for cattle (Young et al., 2003).

The common bean (*Phaseolus vulgaris*) is an important species in the world economy (Parreira et al., 2016), so to increase the profitability of this crop, our knowledge of the molecular mechanism that regulates the seed, the part that is usually consumed, must be further expanded. To study some of these molecular mechanisms, such as regulation of gene expression, it would require the transformation of *P.vulgaris,* which is time consuming and difficult. Until recently, there were no cases of successful transformation of *P. vulgaris* by *Agrobacterium tumefaciens* (G. Song et al., 2020), so the alternative was to use an appropriate molecular model that was easier to transform (Araújo et al., 2004).

Medicago truncatula (M. truncatula), a legume, also known as barrel medic, is commonly used for the study of molecular biology of legumes. With a relatively small genome, a life cycle of approximately 9-10 weeks (Barker et al., 2006) and the fact that the information acquired can be transposed to other economically important legumes, such as *P. vulgaris*, makes *M. truncatula* a solid choice as a plant model for legumes (Choi et al., 2004). Other advantages include: the whole genome is sequenced (Version Mt4.0), and is widely used as a model for nitrogen fixation and symbiosis (Tang et al., 2014). Moreover, in our laboratory there is extensive experience in transforming *M. truncatula* with *A. tumefaciens.* and regenerate it through somatic embryogenisis, more specifically with the embryogenic line M9-10a, that is amenable to transformation (Araújo et al., 2004).

Seed Development

Seed development has 2 major phases: embryogenesis and maturation. The process of embryogenesis begins after a mature pollen grain germinates on a mature stigma and the pollen tube grows through the stigma to reach the ovary and deliver 2 sperm nuclei to the ovule. From these 2 pollen nuclei, one unites with the egg cell to form a zygote that will develop into an embryo and the suspensor that will supply with nutrients and support the embryo. The second nucleus fuses with the central cell (specialized cell in the embryo sac with 2 nuclei) to form a triploid cell that will develop into the endosperm. During the embryogenic development, the embryo changes its morphology and goes through the stages of globular, heart, torpedo, walking stick and mature embryo. In the globular stage, the longitudinal apical-basal axis starts to differentiate and primordial tissue layers start forming. The next stage, heart, the cotyledons start developing in

the apical region and the hypocotyl in the axis. For the next stages the organs grow and accumulate nutrients until maturity is reached. Characteristics such as seed coat pigment, acquisition of desiccation tolerance and inducted dormancy are generally acquire at later stages of development (Goldberg et al., 1994; Martin et al., 2012).

Gene expression regulation

The expression of genes associated with seed filling in *plants* can be regulated at different stages of expression, such as transcriptional, post transcriptional and post translational. During transcription, the association of different transcription factors to a transcriptase influences the expression of a gene, for example. As an example of post transcriptional regulation, small RNAs (sRNA) can induce silencing mechanisms and this is the mechanism that will be mainly focused on. As for post translational regulation, a common example is protein phosphorylation and dephosphorylation (Mazzucotelli et al., 2008).

MicroRNAs (miRNAs)

In recent studies of the development of *P. vulgaris* seeds, several microRNAs (miRNAs) were found to be linked to the role of maintaining seed metabolism and integrity (Parreira et al., 2021). MicroRNAs (miRNAs), are one of several species of small RNAs which consist of RNA that is processed from stem-loop-structured or perfect long double stranded RNAs by Dicer or Dicer-like (DCL) proteins, usually between 20 to 24 nucleotides, that many Eukaryotic organisms use to regulate networks of gene regulatory pathways (Fang & Qi, 2016; Jones-Rhoades et al., 2006). While miRNAs are similar to siRNAs, both chemically and functionally, and used in silencing complexes that include Argonaute proteins, the main difference stems from siRNAs being derived from long double-stranded precursors (Jones-Rhoades et al., 2006). Another interesting characteristic of miRNAs is their conservation between related organisms, opposed to most siRNAs that are not (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee & Ambros, 2001; Reinhart et al., 2002). Some other types of sRNAs include trans-acting small interfering RNAs (ta-siRNAs), heterochromatic siRNAs (hc-siRNAs), heterochromatic siRNAs (hc-siRNAs) long siRNAs (lsiRNAs), long miRNAs (lmiRNAs), double-strand-break (DSB)-induced sRNAs (diRNAs) and DCL-independent siRNA (sidRNAs) (Fang & Qi, 2016).

The miRNAs biogenesis starts with the transcription of the miRNA precursor, a double stranded RNA that in plants is usually found in genomic regions not associated with protein-coding genes (Fang & Qi, 2016; Jones-Rhoades et al., 2006; Reinhart et al., 2002). In plants the miRNA's precursors are processed by DCL1 that cuts the precursor to a miRNA miRNA* duplex with 20 to 24 nt (Fang & Qi, 2016; Jones-Rhoades et al., 2006; Kurihara & Watanabe, 2004) that is methylated by HEN1´ methyltransferase. After methylation the duplex is transported from the nucleus to the cytoplasm by the proteins HASTY and RAN^{GTP} (Jones-Rhoades et al., 2006). Here the miRNAs associate with complexes that contain Argonaute proteins to form the RNA induced silencing complex (RISC) and are able to downregulate the mRNAs by cutting or capturing the transcripts in order to diminish the translation of proteins (Franco-Zorrilla et al., 2007; Jones-Rhoades et al., 2006). A schematic representation of this process is portrayed in figure 1.1.

The RNA induced silencing complex (RISC)

The RNA induced silencing complex consists in the association of Argonaute proteins with small RNAs (sRNAs), which include the miRNAs, and are loaded by Dicer or Dicer-like (DCL) proteins (Fang & Qi, 2016). As the miRNA's precursors are double stranded, after DCL1 processes the precursor, the DCL1 protein in conjunction with HYPONASTIC LEAVES1 (HYL1) and SERRATE (SE) determine the strand selected (Eamens et al., 2009; Lobbes et al., 2006; Manavella et al., 2012). Argonaute proteins are responsible for the cleavage of mRNA and its number in plants varies from species to species, 10 in Arabidopsis (Morel et al., 2002), 15 in poplar (Zhao et al., 2015) and 19 in rice (Kapoor et al., 2008). For proper function of the complex, different associations of Argonaute proteins are required as different types of Argonaute proteins preferably bind to miRNA depending on 5' nucleotide (Mi et al., 2008; Takeda et al., 2008).

Although this complex has several modes of action, only two will be focused on: Endonucleolytic Cleavage and Translational Inhibition (Fang & Qi, 2016). The Endonucleolytic Cleavage is when the RISC through complementarity attaches to a mRNA and the Argonaute protein cleaves it, so it can't be translated (Fang & Qi, 2016). This cleavage happens between the 10th and the 11th nucleotides of the target miRNA. The translational inhibition happens when the RISC has a miRNA and attaches to the target mRNA, but due to 3 nucleotides in the sequence that are not complementary to the miRNA between the 10th and the 11th nucleotides of the target, instead of cutting the transcript it only binds to it. As the position between the 10th and the 11th is essential for the Argonaute protein to cleave, an insertion here prevents cleavage and as the RISC remains bound to the mRNA, the ribosome is blocked by the RISC when attempting to translate, leading to a downregulation (Franco-Zorrilla et al., 2007; Jones-Rhoades et al., 2006; Wong et al., 2018).

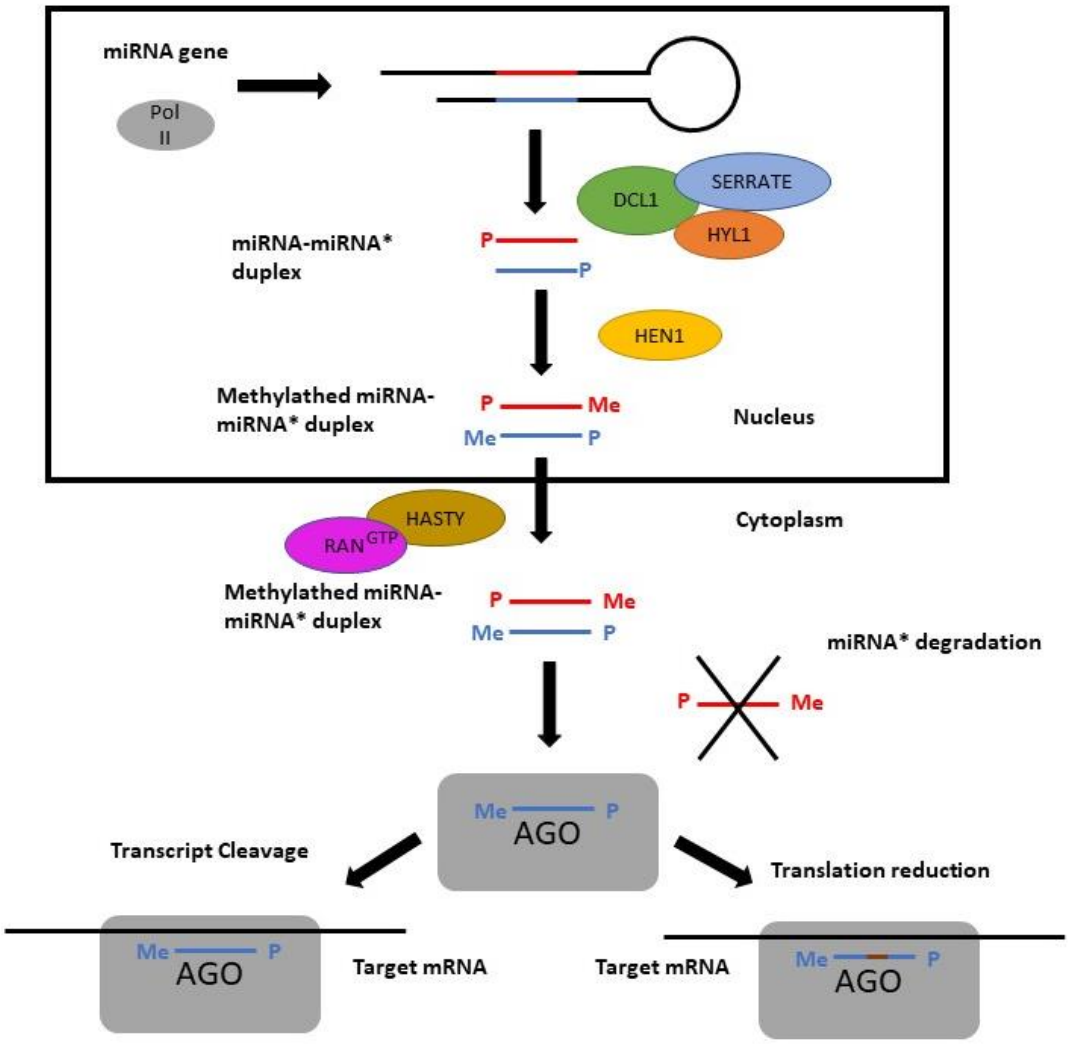

Figure 1.1: Diagram explaining the processing and assembly of miRNA on RISC. After the transcription of the miRNA precursor, the precursor is processed by group of proteins that include SERRATE, HYPONASTIC LEAVES1 and DCL1. This originates a miRNA duplex that is methylated (Me) by HEN1 and then transported to the cytoplasm by HASTY and GTP-binding nuclear protein Ran (RAN^{gtp}). The duplex is assembled in an Argonaut protein and only one of the strands is chosen while the other is degraded. Depending if the miRNA has a bulge between the 10^{th} and 11^{th} nucleotide (brown segment) or not, it can only impede the translation of the target mRNA by binding into it or the RISC can cleave the target (right and left options respectively) .(adapted from Jones-Rhoades et al., 2006)

miRNAs role in seed development

Several studies show that microRNAs have a major role in seed development, with several examples of miRNAs affecting it. The miRNA 156 is known to repress the transcripts of Squamosa Promoter-Binding Protein-Like (SPL) transcription factors during the early stages of embryogenesis to prevent the expression of genes normally found in later stages of development (Martin et al., 2012; Nodine & Bartel, 2010). Another example is miR160 in *Arabidopsis*, that cleaves the Auxin Response Factor17 (ARF17). ARF17 is a transcription factor that when it accumulates in the embryo, it induces trilateral or quadrilateral cotyledons instead of bilateral like in plants where miR160 prevents ARF17 overexpression (Mallory et al., 2005; Martin et al., 2012). Some miRNAs are only known to express in seeds such as miR319a/b/c/d and miR1690 in maize

(*Zea mays*) (Kang et al., 2012). In a brief way, figure 1.2 shows some of the most important miRNA during the development of the seed (adapted from Gupta et al., 2017; Martin et al., 2012).

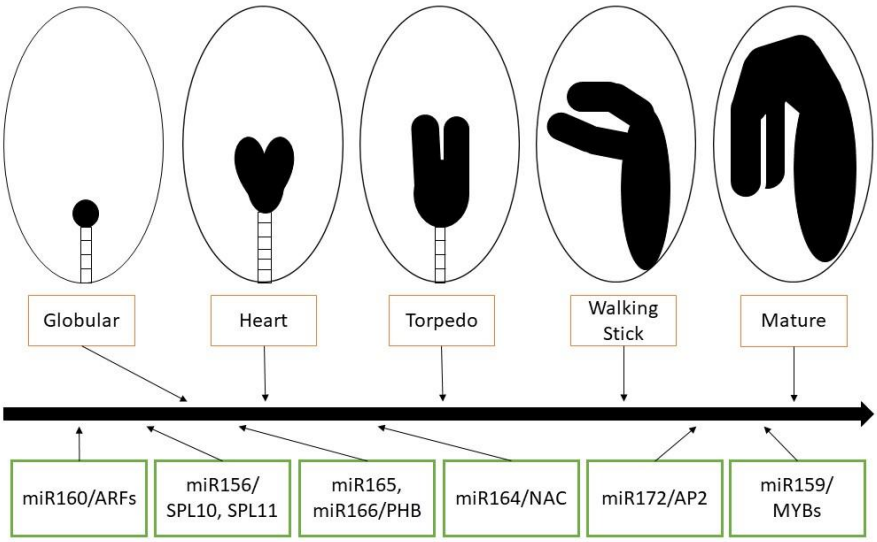

Figure 1.2: Diagram of the stages of seed development and some major miRNAs that regulate this development. miR160 regulates ARFs during the transition from 2 to 4 cells. miR156 regulates SPL 10 and SPL11 in the eight-cell stage. miR65 regulates PHABULOSA (PHB) during globular stage. miR164 regulates NO APICAL MERYSTEM (NAC) during the cotyledon formation between heart and torpedo. miR172 regulates APETALA2 (AP2) and miR159 regulates MYELOBLASTOSIS proteins (MYBs), both are expressed during maturation.

miRNA 408 and its targets

The microRNA 408 is expressed mainly in flowers and in seed pods and differentially expressed in water deprived conditions in *M. truncatula* (Trindade, 2012). Plantacyanin (PLC) transcripts or transcripts of Plantacyanin-like proteins are known to be its target, and it has been confirmed in *A. thaliana, O.sativa* and *M.truncatula* (Abdel-Ghany & Pilon, 2008; Trindade, 2012; Zhang et al., 2017). The respective proteins of these transcripts have copper in its composition, so when there is a shortage of copper due to a reduced nutrient absorption, generally caused by water deficit, miR408 is upregulated in order to diminish plantacyanin expression and thus increasing the copper available for the plant, as less copper is captured in PLC (Abdel-Ghany & Pilon, 2008; Trindade, 2012). The PLC is associated with pollen germination, as a higher expression of this protein can lead to abnormal pollen germination on stigma due to a thinner intine (Dong et al., 2005; Zhang et al., 2018). The pollen intine affects the pollen tube elongation and it has been shown that in PLC overexpression lines the intine can be thinner or form callose formations and generally these pollen have a lower fertility rate and thus less seeds are formed (Dong et al., 2005; Zhang et al., 2018). This lower seed yield is likely not related to defective gametogenesis or defective seed germination as in both cases a PLC overexpression did not change in comparison to wildtype (Trindade, 2012; Zhang et al., 2018). Reports of lower seed yield in plants transformed to downregulate miR408 have been found in *M.truncatula* and *O.sativa,* while the overexpression of miR408 in *A. thaliana* lead to an increase in yield (Trindade, 2012; Song et al., 2018; Zhang et al., 2018).

miRNA 166 and its targets

The microRNA 166 in *A. thaliana* is known to be involved in the cleavage of class III HD-ZIP TFs, such as, PHABULOSA (PHB), PHAVULOTA (PHV) and REVOLUTA (REV). These transcription factors are important to establish the leaf abaxial/adaxial patterning in the embryos and a disruption of complementary region of miR166 leads to the formation of adaxialized leaves (Juarez et al., 2004; Kidner & Martienssen, 2004). The miR166 is also involved in the response to cold stress as *PHB* and *ATHB-8* (*At4g32880*), another possible target of miR166, are involved in this response (Zhou et al., 2008). In *M. truncatula*, miR166 is known to target transcripts encoded for the class-III homeodomain-leucine zipper (HD-ZIP III) such as MtCNA1, MtCNA2 and MtHB8, which are involved in the development of the vascular bundle by controlling the lateral root and nodule formation. An overexpression of miR166 leads to a reduction of the number of lateral roots and symbiotic nodules formed (Boualem et al., 2008).

miRNA 399 and its targets

The miRNA family miR399, is a well-studied family in various types of species, including *M. truncatula,* and it is important in the regulation of inorganic phosphate (Pi) uptake. These miRNAs act by targeting the transcript of the protein PHOSPHATE2 (PHO2), an E2 ubiquitin-conjugating enzyme which represses the Pi uptake (Bari et al., 2006). When the plant faces Pi deprivation, there is a strong induction of miR399 which in turn leads to the cleavage of PHO2 and thus preventing the Pi repression. Upon reintroduction of Pi, the expression of miR399 is greatly reduced. The most likely candidate behind this regulation is the MYB TF PHOSPHATE STARVATION RESPONSIVE1 (PHR1) as proposed by Bari et al. (Bari et al., 2006). Another way to regulate the miR399 is the non-protein encoding transcript INDUCED BY PHOSPHATE STARVATION1 (IPS1), which is known to act as an antagonist that binds to miR399/RISC but cannot be cleaved due to a bulge in the region essential for cleavage (Franco-Zorrilla et al., 2007). As IPS1 cannot be cleaved and maintains to be bound to miR399a/RISC it inhibits miR399a/RISC from cleaving its target PHO2 and thus there is an accumulation of this protein.

Changing IPS1 for target mimicry

Target mimicry is a technique developed to inhibit a specific miRNA from cleaving its target protein. The non-protein encoding gene *IPS1* from *A.thaliana*, has a transcript that shares a region with extensive complementarity with miR399 however, it has a mismatch of 3 nucleotides between the complementary 10th and 11th nucleotides of miR399. As explained before, a mismatch here prevents the cleavage of the transcript, but it will still permit the binding between transcript and RISC. Due to this property the IPS1 transcript acts as a mimic that suppresses the cleavage of PHO2 by reducing the available RISC/miR399 (Franco-Zorrilla et al., 2007). With these evidences, Franco-Zorrilla et al. (2007) theorized that a change in the complementary region of *IPS1,* to be complementary to other miRNAs while maintaining a bulge in the cleavage region, could lead to the development of a technique that could be used as a target mimicry for different miRNAs. This technique has been extensively used in *A.thaliana* (Reichel et al., 2015; Todesco et al., 2010) and this mechanism has also been adapted to other plants such as *O.sativa* (Wang et al., 2015) and *M.truncatula* by introducing the modified *IPS1* assembled with a different promoter through plant transformation (Trindade, 2012).

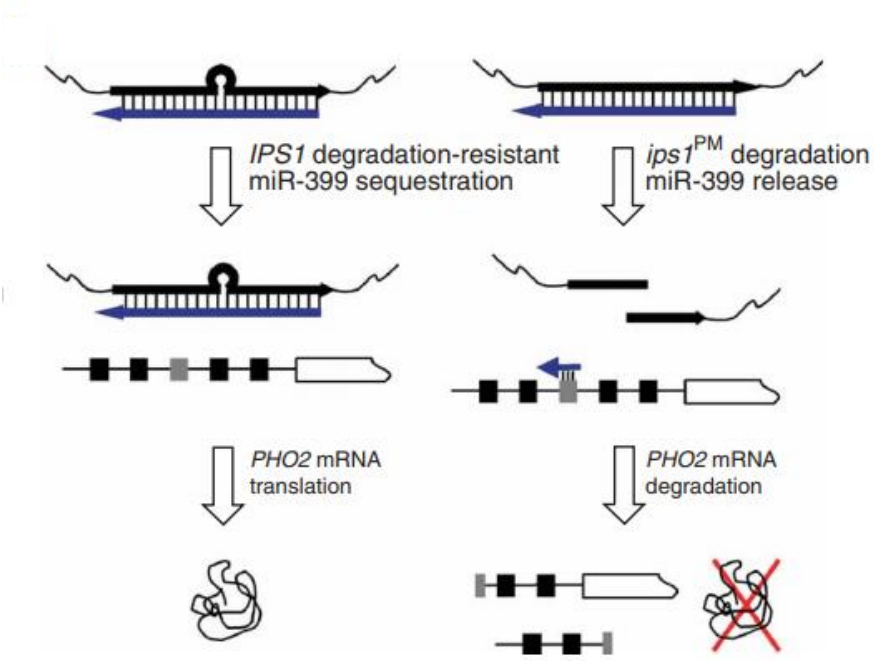

Figure 1.3: Model for miRNA inhibition by target mimicry. The target mimic, represented here by IPS1, requires miRNA recognition (that is, sequence complementarity) as well as resistance to miRNA-guided cleavage (mismatch between the 10th and 11th nucleotides). A degradation-sensitive substrate does not show any significant miRNA inhibitory activity, as measured on a second substrate (PHO2). Image source: (Franco-Zorrilla et al., 2007)

miR408, miR166a and miR399a in *P. Vulgaris*

Based on the recent study of miRNAs in *P. Vulgaris* (Parreira et al., 2021) the miR408, miR166a and 399a were chosen to be studied in *M.truncatula* using the target mimicry technique. Pvu-miR408c (same sequence of miR408 in *M.truncatula*) was chosen because it showed accumulation during the initial stages of seed development and then its abundance decreased significantly in seed tissues (Parreira et al., 2021). It is also known in *M.truncatula,* that miR408 when downregulated leads to lower seed yield (Trindade, 2012) so it is likely that during the first stages of development there will be a contrast of expression between control and transformed plants. Another reason to choose this miRNA was, in the laboratory, there was already a transformed plant with a mimic for this miRNA (Mtr-MIM408) so the expression values of the miR408 and its target (Plantacyanin) could be studied right away. Because the expression of miR166a in *P. Vulgaris* was always present and in great abundance throughout the whole seed development and it is believed to be involved in embryo morphogenesis and the activation of seed maturation (Parreira et al., 2021), it was predicted that a downregulation of this miRNA would lead to phenotype change in the seeds. Finally, miR399a also targets PHO2 similarly to other plants (Bari et al., 2006), and during seed development it accumulated until the seed started to dehydrate, after which it started to decrease. Because PHO2 is associated with phosphorus allocation (Bari et al., 2006), downregulating miR399a would lead to a repression of Pi uptake it

is predicted that it would lead to Pi starvation of the seed similar to what happened in roots of *A.thaliana* when miR399a was downregulated (Franco-Zorrilla et al., 2007).

Objectives

In *P. vulgaris* 3 microRNAs (miR408, miR399a and miR166a) are known to be involved in the development of the seeds so to discover the impact of these miRNAs have on the development of legumes seeds these miRNAs will be studied in *M.truncatula*. In addition, in our laboratory, there were already some studies regarding miR408 in *M.truncatula,* so its targets are already known and there is even a transformed plant (Mtr-MIM408) with a mimic (MIM408) that inhibits the activity of miR408.

The first goal is to identify the homologous miRNAs in *M.truncatula* and its targets. To do this, a search in miRBase was made and a blast tools were used to find the target transcripts of these miRNAs.

The second goal was to observe the role of miR408 in the development of the seed, so to see the effects of miR408 inhibition the transformed plant Mtr-MIM408 was used. In this experiment, the expression of Plantacyanin, MIM408 and miR408 were measured during 5 stages of development of the seed. Plantacyanin (PLC) is the target of miRNA408 and is believed to be involved in pollen germination while MIM408 inhibits miR408 by binding to it and thus preventing the cleavage of PLC by miR408.

The last goal was to create new transformed plants for miR166a and miR399a by mutating the IPS1 gene to be partially complementary to these 2 miRNAs and develop 2 transformed plants containing a mimic of its respective miRNA. The transformation follows a similar protocol to the one used for obtaining Mtr-MIM408.

Material and Methods

Bioinformatic analyses

The miRBase (version 22) (Griffiths-Jones et al., 2006, 2008; Griffiths-Jones, 2004; Kozomara et al., 2019; Kozomara & Griffiths-Jones, 2011, 2014) was searched to retrieve all of the known members of 166 miRNA familiy and miRNA 399a in *M. truncatula*. These were compared to the information available for the pvu-miR166 family and pvu-miR399a from *P. vulgaris* (J. Parreira et al., 2021). The Jalview neighbour joining tree tool (version 2.11) (Waterhouse et al., 2009) was used for this purpose. . After, the tool psRNATarget: A Plant Small RNA Target Analysis Server (2017 update) (Dai et al., 2011, 2018; Dai & Zhao, 2011) was used to conduct a search to identify the predicted targets of mtr-miR166 family, mtr-miR399a and mtr-miR408.

For primer construction, the tools IDT OligoAnalyzer (Integrated DNA Technologies, Inc., Coralville, IA, USA) and NCBI's BLAST (NCBI, National Library of Medicine, USA) were used. For the two-tail primer construction, the RNAfold WebServer (University of Vienna, Institute for Theoretical Chemistry, Austria), was used to determine the secondary structure of the primer. All primers were made by STABVIDA (STAB VIDA, Lda., Caparica, PT). A detailed explanation of each primer created is found in the subsequent subtopics.

Gel Electrophoresis

All electrophoreses were run in 0.5X Tris-Borate-EDTA Buffer (TBE 10X solution 890mM Tris Base, 890mM Boric acid, 20mM EDTA) using SYBR Safe (ThermoFischer, Waltham, MA, USA) as the gel stain. The images were acquired in Gel Doc XR+ Gel Documentation System and visualized in Image Lab Image Capture and Analysis Software (Bio-Rad Laboratories, Hercules, CA, USA).

Plasmid Extraction and digestion

In order to obtain the target mimics of the studied miRNAs (MIM166a and MIM399a), plasmid PDoNR IPS1 was used as origin. Two other plasmids were also extracted, PDoNR/MIM408 that was used as control in vector construction and pk7WG2.0, a plasmid used for the construction of plant transformation vectors through the recombination of a lethal gene (ccdB in pk7WG2.0) with the desired insertion. All plasmids were provided by courtesy of Inês Trindade. The first plasmid contains an IPS1 gene sequence associated to a CaMV 35S promoter and PDoNR/MIM408 has an already modified IPS1 sequence with a region complementary to the miRNA 408. Both plasmids have the IPS1 gene flanked by recombination sequences (attL borders). The third plasmid required is pk7WG2.0, which in a later step will recombine with IPS1, is explained in detail in vector construction section. These PDoNR plasmids were extracted from the *Escherichia coli* strain DH5α and pk7WG2.0 from DB3.1™ E. coli strain, using the NZYMiniprep kit (NZYTech, Lisboa, PT). First, an aliquot of 10 µl from the stock's bacteria were added to 100 ml flask containing 40 µl of LB medium and grown for 12 h at 200 rpm 37 ºC. Depending on the plasmid intended to extract 50 µg/ml of Kanamycin (PDoNR), or 100 µg/ml Spectinomycin (pk7WG2.0). From this culture, 1,5 ml were put in 2 ml tube and centrifuged for 30s at 20238 g. All other centrifugation steps were made with the same acceleration except when

otherwise mentioned. The supernatant was removed and another 1,5 ml of culture were added to the tube and centrifuged for 30 s. The pellet was resuspended in 250 µl of buffer A1 by vortexing and 250 µl of A2 buffer were added to the tube and mixed by inverting it for 8 times. The tube was incubated for between 3 to 4 minutes at room temperature and added 300 µl of A3 buffer and mixed by inverting the tube 8 times. The tube was then centrifuged for 10 minutes and the supernatant was put on a column in a 2ml collecting tube and centrifuged for a minute at 11000 g. The flowthrough was discarded and 500 µl of AY buffer were added and centrifuged. Again, the flowthrough was discarded and 600 µl of A4 buffer were added to the column and centrifuged for a minute and the flowthrough discarded. Another centrifugation was made to dry the matrix for 2 minutes and the column was put in a tube for storage and added 30 µl of AE buffer. The tube was incubated for a minute and centrifuged for a minute. This step was then repeated and the tube containing the purified plasmid stored.

The concentration of the plasmid was then measured in Nanodrop ND-2000C (ThermoFischer, Waltham, MA, USA). To confirm the authenticity of the plasmid, PDoNR IPS1 was then cut with enzyme restriction by EcoRI (ThermoFischer, Waltham, MA, USA) in the following reaction: 16 µl of nuclease free water, 2 µl of 10X buffer EcoRI, 1 µl of plasmid, and 1 µl of EcoRI and incubated overnight at 37ºC. For enzyme inactivation, the sample was incubated 20 minutes at 65ºC. To confirm PDoNR IPS1 extraction the cut plasmid was loaded in an agarose Gel 0.7% (figure 3.2).

Vector Construction

After the extraction of PDoNR IPS1, to change the IPS1 gene to be complementary to miRNA 166a and 399a, a site directed mutagenesis was performed on PDoNR IPS1. The site directed mutagenesis was done according to Hallak et al.(2017) using the primers shown in table 2.1. The M13 primers were initially used to clone the IPS1 gene flanked by attL borders and since the primers were phosphorylated, a ligation was made to circularize the fragment. The ligation was made with T4 DNA ligase (ThermoFischer, Waltham, MA, USA) for 1 hour at room temperature with the following mixture, 10-50 ng of linear DNA, 10x T4 DNA Ligase buffer 5 µl, T4 DNA ligase 5 u and made the total volume to 50 µl by adding nuclease free water. With the fragment circularized the mutagenic primers were used to introduce the desired mutations in sequence make a sequence that is complementary to miRNA 166a and 399 with the exception of 3 introduced nucleotides between the 10th and 11th. This new fragment was also circularized and another PCR was made with the M13 primers to obtain more copies of the fragment and to linearize it again. The fragments as well as PDoNR 408, were sent to sequencing in STABVIDA (STAB VIDA, Lda., Caparica, PT) (supplementary figures 6.1 to 6.6).

All PCR reactions were made using CloneAmp HiFi PCR Premix (Takara Bio, Kusatsu, JP) with the following mixture 12,5 µl CloneAmp HiFi PCR Premix; 0.75 µl for each primer at 0.3 µM (final concentration); 1 µl of template; 10 µl of nuclease free water for a total volume of 25 µl and used the following conditions 98ºC 10 s; 55 ºC 15 s (M13 primers) or 60 ºC 15 s (mutagenic primers) (table 2.1); 72 ºC 10 s for 35 cycles.

Unlike the method used by Hallak et al.(2017), the Gateway technology system (ThermoFischer, Waltham, MA, USA) was chosen to insert the fragment into the desired plasmid through recombination instead of restriction digestion (figure 2.1). Using the gateway technology, the fragments MIM166a and MIM399a, obtained from site directed mutagenesis, and MIM408, from plasmid PDoNR 408, were then inserted in a binary vector named pK7WG2,0 using the LR recombination reaction. These new plasmids were used to transform *E. Coli* strain DH5α. The Gateway system uses the following procedure, 30 ng of the mimic fragment were mixed in 1,5 ml

tube in a volume up to 10 µl, 2 µl of pk7WG2 (300 ng), 4 µl of 5X LR Clonase™ Reaction Buffer and added TE Buffer, pH 8.0 to a final volume of 16 µl. After thawing, 4 µl of LR Clonase™ enzyme mix was added to the tube and incubated at room temperature for an hour. After the incubation, 2 µl of Proteinase K were added and the tube incubated for 10 minutes at 37 ºC followed by a transformation protocol.

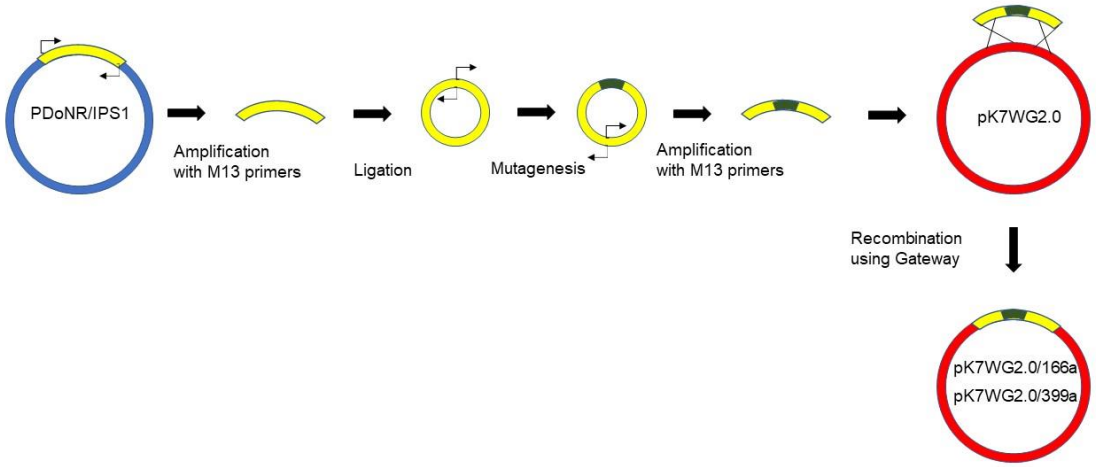

Figure 2.1: Diagram explaining the process of IPS1 mutation and insertion of IPS1 fragment into pK7WG2.0 through recombination. In blue it is represented PDoNR plasmid, in yellow the IPS1 sequence with attL borders flanking it, in green the region of IPS1 mutated by the forward primer to make it partially complementary to miR166a and miR399a, in red plasmid pK7WG2.0.

Table 2.1: Primers used for IPS1 mutation and amplification

MIM166a	Fw: 5' CCTCTAGAAAGGGGAAUGAAGATACCUGGUCCGAAGCTTCGGTT 3'
	Rev: 5' GAGATAAACAAAACTCGCAGTCTGAATCAGCCTTCTG 3'
MIM399a	Fw: 5' CTAGAAACTGGGCAAATCCTATCCTTTGGCAAG 3'
	Rev: 5' AGGGAGATAAACAAAACTCGCAGTCTGAAT 3'
M13	Fw: 5' GTAAACGACGGCCAG 3'
	Rev: 5' CAGGAAACAGCTATGAC 3'

Underlined sections are complementary to target miRNA with the 3 nucleotides between them to create a bulge and avoid cleavage by RISC.

Preparation of *A. tumefaciens* competent cells

To prepare *A. tumefaciens* strain EHA105 for transformation, the competency of the strain was achieved by inoculating the bacteria (previously stored at -80 ºC in glycerol) in 50ml LB media overnight at 28ºC on a rotating shaker. The culture was chilled in ice for 30 minutes and then 45 ml of culture centrifuged for 10 minutes at 3082 g, 4ºC. The supernatant was removed and the pellet resuspended in 5ml of ice cold 20mM calcium chloride. The culture was centrifuged again at the same acceleration and temperature for 5 minutes and the supernatant was discarded with the pellet being resuspended in 1 ml of 20 mM calcium chloride. Several aliquots were made containing 10% (v/v) glycerol and snap frozen in liquid nitrogen and finally stored at -80 ºC.

Transformation of competent dh5a E. coli

To transform *E. coli* strain DH5α with 3 different binary vectors pK7WG2.0/MIM166a, pK7WG2.0/MIM399a and pK7WG2.0/MIM408, the competent cells were transformed by chemical transformation. The procedure consisted in putting 50 µl of competent cells (originally stored at -80 ºC in glycerol) in 1,5 ml tube and adding 1 µl of one of the three vectors and mix it gently. The tube was then incubated for 30 minutes on ice followed by heat-shocking the cells for 30 s at 42 ºC and immediately transferred to ice. While on ice, 450 µl of S.O.C. (Takara Bio, Kusatsu, JP) at room temperature was added and the tube were incubated and shaken at 200 rpm at 37 ºC for an hour. After incubation, 20 µl and 100 µl of culture were plated in LB agar supplemented with spectinomycin (100 µg/ml).

Plant material and growth medium

Transformed plants (Mtr-MIM408 – experimental group and Mtr-GFP – control group) originally of the M9-10a genotype, *M. truncatula* Gaertn cv. Jemalong, were the main study material. All plants were grown and maintained similar to Neves et al. (2001), in growth-regulator-free medium MS030A-MS (Murashige & Skoog, 1962) with vitamins and basal salts, 2% (w/v) sucrose, 0,7% micro agar (Duchefa, Haarlem, NL). The medium had its pH adjusted to 5.8 before autoclaving (121 ºC, 20 min) All cultures were maintained in a growth chamber (PHYTOTRON EDPA 700, ARALAB) with 16-h photoperiod of 100µmol m−2 s−1 applied as cool white fluorescent light and a day/night temperature of 24 ◦C/22 ◦C.

Greenhouse plant growth and seed and pod collection

Regarding the growth of *M. truncatula,* the plants were grown *in vitro* for roughly a month before being transferred to jiffy pellets (Jiffy International AS, Kristiansand, NO) and maintained in the growth chamber for 3 weeks in the same light and temperature conditions as in vitro plants. After this period the plants were transferred to pots containing soil mixed with peat and vermiculite using the following ratio (2:1:1) (Compo Sana SA., Barcelona, ES) and grown in the greenhouse. Due to asynchronous flowering the pods were collected throughout the months of March, April, May and June. The collection of pods was based on 5 different morphologies of the pod during development as seen in figure 2.2. The seeds were collected and stored at a temperature of -80 ºC based on 5 growth stages of the seed. As the first and second stage seeds are too small to be separated from the pod, both the pod and seed were stored. For the remaining stages, only the seeds were stored. Besides seeds, leaves were also stored in -80 ºC, as material to check for the presence of MIM-408 in Mtr-MIM408 and M9-10a plants. Each of these stored samples was

grounded together to a fine powder in liquid nitrogen using a chilled mortar and pestle and stored roughly 0.1g of material in a 2 ml tube.

Figure 2.2: Representation of a floral apparatus and the five stages of pod development. It should be noted that the different stages were chosen by phenotype observation and not on a temporal scale. The distance between lines is 8 mm.

RNA Extraction

For obtaining RNA from leaves a manual extraction was performed. This extraction used 900ul of RNA extraction Buffer (2% (w/v) CTAB; 2% (w/v)PVP; 100mM Tris HCl pH 8; 30mM EDTA pH 8; 2M NaCl; 0.5g/l spermidine; 3% (v/v) B-mercaptoethanol) and incubated for 30 minutes at 65 ºC. This was followed by 2 consecutive extractions with 900 ul of chloroform: isoamyl alcohol (24:1) by centrifuging 15 minutes at 12000 g and only the superior phase was collected. To this solution LiCl was added to a final concentration of 3M to precipitate the RNA. The new solution was incubated for an hour at -20 ºC and after that centrifuged for 20 minutes at 20238 g. The supernatant was discarded and the pellet was washed two times with 600 ul 70% ethanol at -20 ºC and with 600ul 100% ethanol once. After each wash, the tube was centrifuged for 3 min at 20238 g. The pellets were then left to air dry for 5 minutes and diluted in 40 ul of cold mili-Q water RNAse free water. The RNA was put on ice for 5 minutes before measuring concentrations with the spectrophotometer Nanodrop ND-2000C (ThermoFischer, Waltham, MA, USA).

To ensure that the RNA extracted is free of DNA contamination, 20 ul of the RNA extracted was mixed with 2ul of buffer and 1 ul of Ambion DNAse Turbo (ThermoFischer, Waltham, MA, USA) and incubated 15 minutes at 37ºC. To stop this reaction 2ul of DNase Inactivation Reagent was added and the solution incubated for 2 minutes at room temperature and centrifuged for 1,5 minutes at 10000g. Finally, the supernatant containing now the purified RNA was retrieved and the RNA was analysed in an electrophoresis gel (2%) to assess its integrity.

The Spectrum Plant Total RNA kit (Merck KGaA, Darmstadt, DE) was used to extract roughly 0.1 g of the seed's powder per sample. Initially the tubes with the frozen powder were placed on ice and 500 µl of Lysis solution and 5 µl of β-mercaptoethanol were added to each sample and vortexed for 30 seconds. The samples were incubated at 56 ºC for 4 minutes and then centrifuged at 20238 g, all subsequent steps were centrifuged at the same acceleration. The supernatant was retrieved and put on a filtration column to centrifuge for a minute. To the flow-through, 500 µl of binding solution were added and mixed. Of this new solution 500 µl were put

in a binding column, centrifuged and the flow-through discarded. Using the same column, the last step was repeated with the remaining solution. To the binding column 300 µl of Wash solution 1 were added, centrifuged for 1 minute and the flow-through discarded. A mix of 70 µl of reaction buffer and 10 µl of DNAse were added to the binding column and incubated at room temperature for 15 minutes. To the binding column 500 µl of Wash solution 1 were added, centrifuged for 1 minute and the flow-through discarded. With the same binding column empty, it was centrifuged again and then 500 µl of Wash solution 2 were added. The column was centrifuged for 30 seconds, the flow-through discarded and again centrifuged for a minute. The column was placed in a tube intended for storage and added 50 µl of elution solution and then incubated at room temperature for 1 minute. Finally, the column was centrifuged for a minute and the purified RNA was stored.

The RNA extracted was later analysed by spectrophotometry using Nanodrop ND-2000C to assess its concentration and quality (table 6.8). To determine the RNA integrity, it was first denatured in formamide buffer (10 ml of dionized formamide, 200ul 0,5M EDTA pH8.0, 10mg xylene cyanol, 10mg bromophenol blue) at 65ºC for 10 min. Then, an aliquot of RNA was run in agarose gel (2%) (supplementary figures 6.7 and 6.8). To determine if the RNA was free of DNA contamination, an amplification of an aliquot using Ubiquitin intron primers (table 2.5).

cDNA Synthesis

The cDNA from the target, MIM408 and reference genes was synthesized using ImProm-II™ Reverse Transcription System (Promega,Madison, WI, USA) in a total volume of 20ul, using 0,5 µg of RNA template by using the oligo dT. The detailed procedure is as following, in a 1,5 ml tube using 0,5 µg of RNA template were added with 1 µl of oligo dT primer (provided by the kit) and enough nuclease water to make up a total volume of 5 µl. The tube was incubated at 70ºC (pre-heated) for 5 minutes and immediately placed on ice for 5 minutes. After, a short spin was made in the centrifuge to collect the condensate. Another tube, of 50 µl, was prepared with 4 µl of ImProm-II™ 5X Reaction Buffer, $MgCl_2$ 3 µl,1 µl dNTP mix, 1 µl ImProm-II™ of Reverse Transcriptase and 6 µl of nuclease free water to a total volume of 15 µl. On ice, both tubes were mixed to a final volume 20 µl and incubated at room temperature for 5 minutes. Using a thermal cycler, the tube was incubated at 42ºC for 55 minutes followed by 15 minutes at 70 ºC and the tube was stored.

Using the Two-tailed method described by Androvic et al, the cDNA from miRNA 408 was synthesized using qScript® Flex cDNA Synthesis Kit (Quantabio, Beverly, MA, USA) with the Two-tailed primer that can be found in table 2.4 and 0,5 µg of RNA template. The procedure followed these steps, in a 50 µl tube 0.5 µg of RNA template was mixed with 0.5 µl of qScript reverse transcriptase, 2 µl of qScript Flex Reaction Mix, 1 µl of GSP Enhancer, 1 µl of the two-tailed primer in a final concentration of 1 uM and added nuclease free water to a final volume of 10 µl. The tube was gently vortexed and incubated at 25 ºC for 45 minutes, followed by 5 minutes at 85 ºC, in a thermal cycler. Before storing the tube, it was put on ice for 5 minutes.

PCR amplification

To determine if Mtr-MIM408 still expressed MIM408, the cDNA obtained from leaf RNA was amplified by PCR. The primers used in this reaction were MIM408 primer found in table 2.2. The PCR protocol is the following: 5 µl of 5X Green GoTaq® flexi buffer, 2 µl of $MgCl_2$,1 (25mM) µl of GoTaq® G2 Flexi DNA Polymerase (5 u/µl) diluted 8 times, 0,5 µl of each primer (25 µM),

1 µl of dNTPs mix (5mM), template DNA and added nuclease free water to a final volume of 25 µl. The PCR program had an initial 2 minutes denaturation step at 95ºC followed by 30 cycles of 95ºC for 15 s, 53 ºC for 15 s, 72ºC for 30 s and a final extension of 5 minutes at 72ºC. This protocol, with minor changes, was also used to search for MIM408 contaminations and perform cPCR. In cPCR instead of using template DNA, bacteria picked from a sterile toothpick were put in the PCR solution. To check for MIM408 contaminations in seed and pods cDNA, either MIM408 primers (table 2.2) or MIM408n primers (table 2.5) were used.

RT-qPCR

The objective of the RT-qPCR assay was to determine if the abundance of plantacyanin (target), miR408 and MIM408 (mimic) varied from Mtr-GFP to Mtr-MIM408 plants in determined stages of seed and pod development.

RT-qPCR reactions for the target and the mimic were performed using PerfeCTa ® SYBR® Green SuperMix (Quantabio, Beverly, MA, USA) in a total volume of 20 µl in the lightCycler® 480 Instrument II (Roche Holding AG, Basel, CH). For this reaction, 10 µl of PerfeCTa SYBR Green SuperMix (2X) was mixed with the forward and reverse primers (each in final concentration of 300 nM), template and nuclease free water up to 20 µl. A 96 well was used for the reactions with the following settings: incubation for 30 s at 95ºC, followed by 45 cycles of 95ºC for 5 s and 60ºC for 15 s. Dilutions curves were performed to assess the efficiency of the pairs of primers and the total cDNA used was 10 ng, 5ng, 1 ng and 0,1 ng (assuming 100% of RNA was reverse transcribed to cDNA). The primers used to amplify the cDNA from Plantacyanin and MIM408 can be found in table 2.2.

For miR408 detection the same procedure was used but a total volume of 10ul was used instead. The primers used to amplify miR408 in table 4.

According to the literature, the genes found in table 2.3 (GAPDH3, PDF2, PPRep, Mtc27, Aprt, L2) were tested to see which ones were the most suitable as reference genes using GenEx 7.0 (MultiD Analyses AB,Göteborg,SE). The same dilutions curves were performed to assess the efficiency of the 7 pairs of primers.

To calculate parameters such as efficiency (E) and ΔΔCt the following formulas were used, with Gi standing for gene of interest and Rg as reference gene:

$$E = (10^{(-1/\text{slope})}) \times 100$$

$$\Delta Ct = \text{Average Ct of Gi} - \text{Average Ct of Rg}$$

$$\Delta\Delta Ct = \Delta Ct \text{ of Target} - \Delta Ct \text{ of Control}$$

$$\text{Fold difference } 2^{-\Delta\Delta Ct}$$

Table 2.2: Primers used for Plantacyanin and MIM408 detection in RT-qPCR

Gene	Primer	Amplicon size	Efficiency	Reference
Plantacyanin	Fw: 5' GGAAGAGGCAGTGCATTGG 3'	153 nt	stage 1: 1.98	(Trindade, 2012)
	Rev: 5' GGTATCACCAGCCCTAAAGC 3'		stage 5: 1.98	
MIM408	Fw: 5' TGAAGACTGCAGAAGGCTGA 3'	215 nt	stage 1: ND*	
	Rev: 5' CTCACACAAAGAACACACAACG 3'		stage 5: ND*	

*ND: not determined due to no linearity between the Ct values of the dilutions.

Table 2.3: Primers for the reference genes tested in RT-qPCR

Gene	Primer	Amplicon size	Efficiency	Reference
Act2	Fw: 5' TCAATGTGCCTGCCATGTATGT 3'	101 nt	stage 1: 1.97	(Kakar et al., 2008)
	Rev: 5' ACTCACACCGTCACCAGAATCC 3'		stage 5: 1.94	
GAPDH3	Fw: 5' TGCCTACCGTCGATGTTTCAGT 3'	103 nt	stage 1: 1.99	
	Rev: 5' TTGCCCTCTGATTCCTCCTTG 3'		stage 5: 1.98	
PDF2	Fw: 5' GTGTTTTGCTTCCGCCGTT 3'	114 nt	stage 1: 1.97	
	Rev: 5' CCAAATCTTGCTCCCTCATCTG 3'		stage 5: 1.98	
PPRep	Fw: 5' GGAAAACTGGAGGATGCACGTA 3'	112 nt	stage 1: 1.96	
	Rev: 5' ACAAGCCCTCGACACAAAACC 3'		stage 5: 1.97	
Mtc27	Fw:5' TGAGGGAGCAACCAAATACC 3'	93 nt	stage 1: 1.98	(Marino et al., 2011)
	Rev: 5' GCGAAAACCAAGCTACCATC 3'		stage 5: 1.98	
Aprt	Fw: 5' ATGTGCTGCGATTAAACTAC 3'	139 nt	stage 1: 1.94	(Capitão et al., 2011)
	Rev: 5' GGCTCCTTCTCCTTCAAC 3'		stage 5: 1.87	
L2	Fw: 5' GCTTACCACAAGTTCAGAG 3'	164 nt	stage 1: 1.93	
	Rev: 5' GCAATGAGACCAACCTTC 3'		stage 5: 1.91	

Table 2.4: Primers used for miR408 detection in RT-qPCR

	Primer	Efficiency
miR408 specific primers	Fw: 5' ATCCTCTCCAGGTACAGTTGG 3'	stage 1: 1.96 stage 5: 1.97 Shoot: 1.94
	Rev: 5' GTGATGCACTGCCTCTTCCCT 3'	
Two-tailed primer	5' GCAGTGCATCTATCCTCTCCAGGTACAGTTGGTACCTGTCTCG ACTAGCCAG 3'	

The sequence underlined is complimentary to miRNA while the rest of the sequence corresponds to the hairpin-loop.

Table 2.5: Primers used for MIM408 DNA contamination detection.

	Primer	Amplicon size
MIM408n	Fw: 5' GGAAGGCTTGGCAGTGCATA 3'	151 nt
	Rev: 5' ACACAAAGAACACACAACGC 3'	
Ubiquitin Intron	Fw: 5' CTCTAAGGTTTAATGAACCGG 3'	101 nt
	Fw: 5' AGACACAGCCAAGTTGCA 3'	

The sequence underlined is complimentary to MIM408

Results

Bioinformatic

From the comparison between miR399a from *P. vulgaris (*pvu*)* and miR399a from *M. truncatula* (mtr) and miRNA166 in both plants, using the Jalview tool, figure 3.1 was obtained. Regarding the 166 familiy, only 3 miRNAs were fully conserved between *P. vulgaris* and *M. truncatula* based on figure 3.1*.* Additionally, pvT-miR166G and mTr-miR166h are identical with the latter having an extra nucleotide in 3' end. Comparing the miR399a, the 12th and 20th nucleotides are changed as seen in table 3.1.

In order to compare the predicted targets in *P. vulgaris* (J. Parreira et al., 2021), a search was conducted in order to find possible targets for the chosen miRNAs of *M. truncatula* (166 family/399a/408a) and it was obtained using the psRNATarget tool. For the 166 miRNA family all targets were class III homeodomain leucine zipper proteins which was expected, as it had already been described by McHale and Koning, (2004) and Kim et al., (2005). For mir399a, while using the same tool, the initial results were not the expected, as the expected target Ubiquitin-conjugating enzyme (E2 ligase) was not listed, as stated by Bari et al., (2006), Jones-Rhoades (2006) and Jagadeeswaran et al. (2009). After extending the search to NCBI´s Standard Nucleotide BLAST using miR399a sequence, the two alignments with best score were of a predicted protein E2 ligase either on the 2nd or 4th chromosome. By inserting both sequences of the predicted protein E2 ligases found in BLAST tool in the psRNATarget tool, the transcripts of E2 ligase from the 2nd chromosome had a better score than the other transcripts initially listed in psRNATarget tool. For miR408 the same search was conducted in psRNATarget tool and it had plantacyanin (Medtr8g089110) as one of its predicted targets with a score of 1, with score closer to 0 being better and only other potential target had a better score a MYB family transcription factor (Medtr1g086180). This time the target is in accordance to the reported in the bibliography, as Trindade (2012) showed experimentally this protein was the target for miR408.

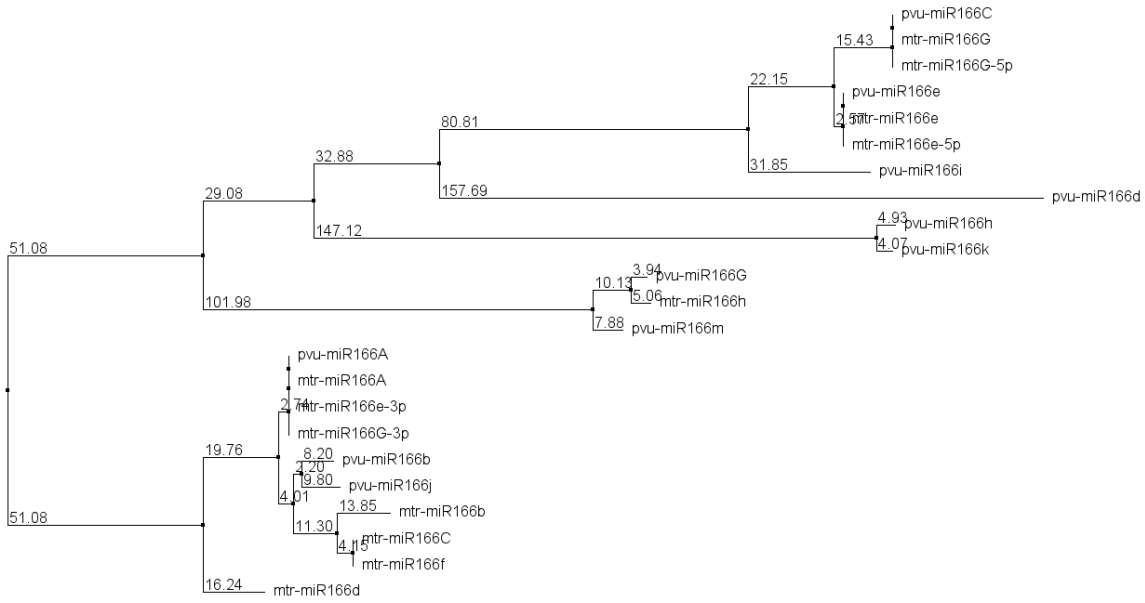

Figure 3.1: Neighbour joining tree of miRNAs from 166 family of both *P. vulgaris* (pvu) and *M. truncatula* (mtr).

Table 3.1: Comparison between miRNAs from *P. vulgaris* and *M. truncatula*

miRNA	Sequence
mtr-miR166A	5' TCGGACCAGGCTTCATTCCCC 3'
pvu-miR166A	5' TCGGACCAGGCTTCATTCCCC 3'
mtr-miR399A	5' TGCCAAAGGAGATTTGCCCAG 3'
pvu-miR399A	5' TGCCAAAGGAGAGTTGCCCTG 3'
mtr-miR408-3p	5' ATGCACTGCCTCTTCCCTGGC 3'

Underlined letters show differences in nucleotides between *P. vulgaris* (pvu) and *M. truncatula* (mtr)

Vector Construction

Before sending plasmid PDoNR/IPS1 and PDoNR/MIM408 for sequencing, there was a confirmation test using restriction enzymes to determine if the extracted plasmids were the desired ones. This confirmation can be seen in figure 3.2

For the vectors' construction pk7wg2, both IPS1 fragments were successfully mutated so that when translated, they can act as mimics for mir166a and 399a. This was confirmed by sequencing the both fragments as MIM399a showed in both strands the intended sequence and in MIM166a the template strand had the intended sequence while the coding one had the 15th and 23rd nucleotides changed from a guanine to an adenine. The plasmid PDoNR/MIM408 was also sequenced and it also had the desired sequence (table 3.2) (for complete sequence see supplementary figures 6.1 to 6.6).

Regarding the Gateway technology system, several attempts to perform the LR recombination reaction with the fragments containing MIM399a and MIM166a and plasmid PDoNR408 with plasmid PK7wg2 were made, but no colony containing the MIM/PK7wg2 Plasmid was obtained. Initially this was due to an oversight of the chosen selection marker, as by mistake, kanamycin was used, but the error was not detected as a colony grew despite the vector conferring resistance to spectinomycin. This colony, supposedly transformed with MIM399/pk7wg2.0 was grown for plasmid extraction in LB solution and tested with cPCR with positive results for the presence of the IPS1 fragment that can be found in figure 3.3. For the cPCR, the MIM408 primers (table 2.2) were used for the detection, as they amplify a fragment of around 200 nt that contains the region complementary to the miRNA of the IPS1 fragment but cannot distinguish between the 3 different mimics. With this result, several attempts to retrieve the plasmid were made with limited result, as very few amounts of plasmid were obtained. This plasmid was sent to sequencing, but it failed. To understand the reason behind the lack of success, all the procedure was reviewed until it was discovered that the problem lied on the usage

of the incorrect selection marker. A new transformation was attempted now with spectinomycin, but no colonies were obtained. As such, without a binary vector, it was not possible to transform plants using *A tumefaciens.*

Table 3.2: Sequence of the mutated IPS1 regions for MIM166a, MIM399a an MIM408. Underlined letters in sequence results highlight nucleotide changes in comparison to the pretended sequence. Underlined letters in the pretended sequence highlight the bulge.

MIM166a sequence result	Fw: 5' GGGGAATGAAAATACCTGGTCCAA 3'
MIM166a pretended	Fw: 5' GGGGAAUGAAGATACCUGGUCCGA 3'
MIM166a sequence result	Rev: 5' TCGGACCAGGTATCTTCATTCCCC 3'
MIM166a pretended	Rev: 5' TCGGACCAGGTATCTTCATTCCCC 3'
MIM399a sequence result	Fw: 5' CTGGGCAAATCCTATCCTTTGGCA 3'
MIM399a pretended	Fw: 5' CTGGGCAAATCCTATCCTTTGGCA 3'
MIM399a sequence result	Rev: 5' TGCCAAAGGATAGGATTTGCCCAG 3'
MIM399a pretended	Rev: 5' TGCCAAAGGATAGGATTTGCCCAG 3'
MIM408a sequence result	Fw: 5' GCCAGGGAAGGCTTGGCAGTGCAT 3'
MIM408a pretended	Fw: 5' GCCAGGGAAGGCTTGGCAGTGCAT 3'
MIM408a sequence result	Rev: 5' ATGCACTGCCAAGCCTTCCCTGGC 3'
MIM408a pretended	Rev: 5' ATGCACTGCCAAGCCTTCCCTGGC 3'

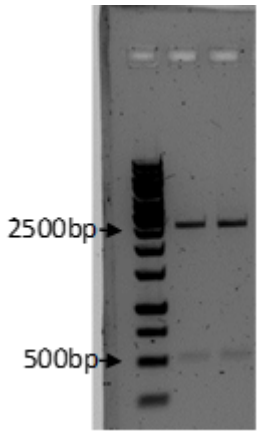

Figure 3.2: Confirmation of PDoNR/IPS1 and PDoNR/408 plasmids. Both plasmids before cutting should have between 3kb and 3,5 kb. After the restriction with EcoRI a 500 bp and a 2500/3000 bp band should appear as the results demonstrate. First well 100kb ladder, second well PDoNR/IPS1; third well PDoNR/408.

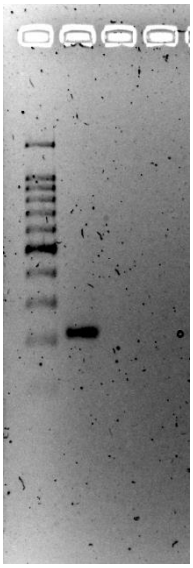

Figure 3.3: Electrophoresis gel (2%) of cPCR products amplified for IPS1/MIM399 fragment that was inserted in pk7WG2.0 binary vector with the following contents from left to right: ladder 100bp; IPS1/MIM399 amplification; negative control. The expected band for IPS1/MIM399 is 215 bp and as the closest band to the fragment in the ladder is 200 bp, it is assumed that colony contains the fragment.

Characterization of plant and seed phenotypes in Mtr-MIM408 and Mtr-GFP plants

During growth both MIM408 transformants and control plants displayed similar phenotypes with the only distinguishable differences being delayed blooming of flowers and fewer number of beans in Mtr-MIM408 in comparison to Mtr-GFP plants. Even though *M.truncatula* has asynchronous flowering, the MIM408 plants started blooming at least 2 weeks later than the control and with fewer flowers. It should also be noted due to restrictions imposed in March 2020 due to the ongoing pandemic, the flowering process could not be daily monitored and so the phenotype distinctions were only observed but not documented. The restrictions also changed the method of collection, as instead of collecting the flowers and seed pods by days after anthesis, it was collected based on its phenotype and were split into 5 different stages as seen in figure 2.2.

Figure 3.4: example of floral apparatus of *M. truncatula.*

Separar rna dos targets e do mirna

Rna Extraction and RT-qPCR Analysis

The RNA from leaves, that was converted to cDNA to confirm the expression of MIM408 in Mtr-MIM408, showed an amplification of the desired size as seen in figure 3.5.

The RNA of stage 1 and 5 was successfully extracted from both control and transformed plant (Mtr-GFP Mtr-MIM408) as was the cDNA synthesis of the conventional and Two-tailed method (figure 6.7). The pair of reference genes that got the best score from GenEx 7.0 (MultiD Analyses AB,Göteborg,SE) were GAPDH3 and PPRep. From the RT-qPCR analysis some unexpected results were found, as the control showed expression of MIM408 (tables 6.5 and 6.6). To understand the reason behind this result, the cDNA from control plants was amplified using the MIM408 primers (table 2.2) and it showed a fragment of over 200 nt (figure 3.6). To make sure it did not contain the MIM408 in its genome, a new set of primers (MIM408n; table 2.5) were designed so that the forward primer was complementary to the modified region of IPS1, the region that binds to miR408. Both the Mtr-MIM408 and Mtr-GFP were tested, and both showed a band with the expected 150bp, though the control also showed an additional band with 500bp (figure 3.7). To conclude if this was due to a mix of Mtr-GFP and Mtr-MIM408 samples or the plants used were unknowingly transformed or if the M9-10a genotype transcriptome contained a sequence that could be amplified, the following procedure was made: RNA extraction of stage 2 seed pods of Mtr-GFP, and of RNA from leaves of plants known to be M9-10a genotype that were in vitro (figure 6.8). With RNA from both samples, cDNA was synthetized, and it was amplified using the MIM408n primers (table 2.5), the forward primer is complementary to the region of MIM408 that

binds to miR408 to guarantee the expression is due to the expression of MIM408 and not from other transcript similar to IPS1. Again, the control sample showed the same 2 bands while the m9-10a genotype showed only the 500bp band (figure 3.8 and 3.9 respectively). It should be noted that the stage 2 pods had not been used up until this point, which means if there was a contamination of the controls, it happened during the collection of the pods, so all samples are contaminated and the experiment is invalid. In an attempt to discover the reason behind the 500bp band appearance, using the BLAST tool, a blast was made using the MIM408n primers, though no fragment with 500 bp was predicted even after considering possible fragments created as off-targets. Even though the control samples showed presence of MIM408, nullifying the possibility of reliable $2^{-\Delta\Delta Ct}$ values, these were still calculated to gather additional information to ascertain the origin of MIM408 in control samples and check for salvageable material (tables 3.3 and 3.4).

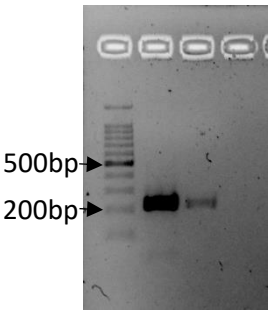

Figure 3.5: Electrophoresis gel (2%) of PCR products amplified for MIM408 from total cDNA from Mtr-MIM408 leaves with the following contents from left to right: ladder 100bp; MIM408 cDNA amplification; MIM408 fragment as positive control, amplification without template as negative control. The expected band for MIM408 is 215bp

Table 3.3.: calculated $2^{-\Delta\Delta Ct}$ of stage 1 (S1) and 5 (S5) using either GAPDH3 or PPRep as the reference gene for Plantacyanin. *Not accounted in the average.

	S1		**S5**	
	GAPDH3	PPRep	GAPDH3	PPRep
ΔΔCt 10 ng	0.283221	0.505226	0.309927	0.243164
ΔΔCt 5 ng	0.27389	0.093861*	0.312083	0.303549
ΔΔCt 1 ng	0.631783	0.654062	0.291688	0.212053
ΔΔCt 0.1 ng	0.54055	1.481953*	0.156855	0.117237
Average	0.432361	0.579644	0.267638	0.219001

Table 3.4: calculated $2^{-\Delta\Delta Ct}$ of stage 1 (S1) and 5 (S5) using either GAPDH3 or PPRep as the reference gene for miR408. ND: not determined due to lack of data or outlier.

	S1		**S5**	
	GAPDH3	PPRep	GAPDH3	PPRep
ΔΔCt 10 ng	0.057711	ND	10.92832	ND
ΔΔCt 5 ng	0.068393	0.108067	7.808254	3.105876
ΔΔCt 1 ng	0.034794	0.070073	11.08088	6.233317
ΔΔCt 0.1 ng	ND	ND	9.713559	ND

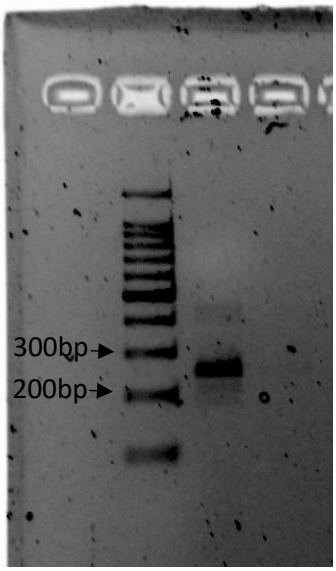

Figure 3.6: Electrophoresis gel of PCR products amplified for MIM408 from total cDNA from pods of Mtr-GFP with the following contents from left to right: ladder 100bp; MIM408; negative control. The expected band for MIM408 is 215bp

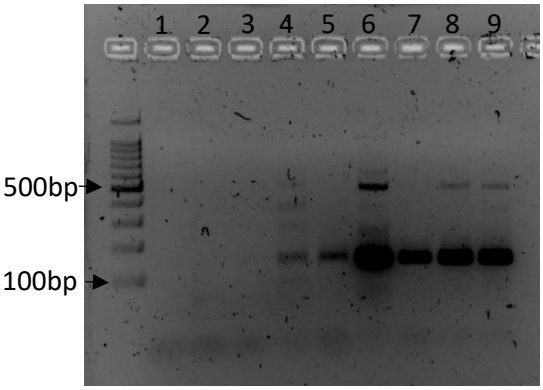

Figure 3.7: Electrophoresis gel of PCR products amplified for MIM408 from total cDNA from pods of Mtr-GFP and Mtr-MIM408 plants with the following contents from left to right: ladder 100bp;1 - negative control; 2 - negative control; 3 - TR S1 A; 4 - TR S1 B;5 - Ctl S1 A; 6 - Ctl S1 A; 7 - TR S5 A; 8 - Ctl S5 A; 9 - Ctl S5 B (TR-Mtr-MIM408; Ctl – Mtr-GFP; S – stage of development). The expected band for MIM408 is 150bp and there is also an unknown band with roughly 500bp.

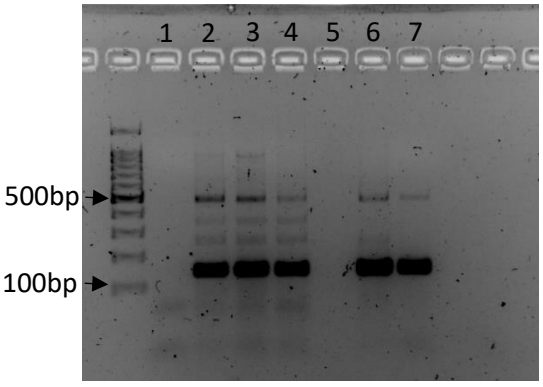

Figure 3.8: Electrophoresis gel of PCR products amplified for MIM408 from total cDNA from stage 2 pods of control plants with the following contents from left to right: ladder 100bp; 1- negative control; 2 – MIM408 S2 A; 3 - MIM408 S2 B; 4 - MIM408 S2 C; 5 - empty well; 6 - positive control; 7 – positive control (S – stage of development). The expected band for MIM408 is 150bp and there is also an unknown band with roughly 500bp.

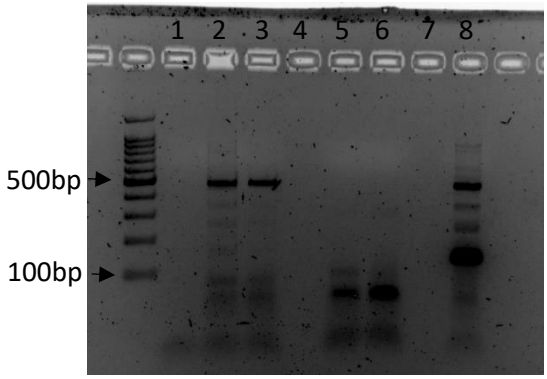

Figure 3.9: Electrophoresis gel of PCR products amplified for MIM408 from total cDNA from leaves of M9-10a genotype plants, with the following contents from left to right: ladder 100bp; 1 - negative control; 2 - MIM408 M9-10a A; 3 - MIM408 M9-10a B; 4 - empty well; 5 - control A; 6 - control B; 7 empty well; 8 - positive control. The expected band for MIM408 is 150bp, there is also an unknown band with roughly 500bp. The control A and B should have been an amplification of one of the reference genes to demonstrate the presence of RNA in case of the samples revealed no band. Instead by mistake the amplification for the ubiquitin intron was used. Because the 500 bp band is present in both samples there was no need to prove the presence of RNA.

Figure 3.6, 3.7, 3.8 and 3.9 use a 2% agarose gel the 100 bp ladder last band represents 100bp and the 5th counting from the bottom is 500 bp. The negative control used were PCR amplifications with no template. Positive controls are MIM408 amplifications from

Discussion

miRNAs and their targets in *M.truncatula*

MiRNAs from *P.vulgaris* were found to be

All of the targets of the miRNAs studied the same as the ones found in bibliography, 166 miRNA family all targets were class III homeodomain leucine zipper proteins (Kim et al., 2005; McHale & Koning, 2004), miR408 the target was plantacyanin (Trindade, 2012) and miR399a targe was E2 ligase (Bari et al., 2006; Jagadeeswaran et al., 2009; Jones-Rhoades et al., 2006). Although the predicted target in miR399a is aligned with bibliography initially the results in psRNATarget tool using Mt4.0v1 library did not list E2 ligase as a target. Since Jagadeeswaran et al. (Jagadeeswaran et al., 2009) predicted in Medicago that the target would be a E2 ligase, an analysis was made in NCBI´s Standard Nucleotide BLAST to verify the targets. This was done by verifying the homologous sequences to the predicted sites of cleavage and the sequence provided by miRBase. In all cases the best scores belonged to a predicted protein E2 ligase either on the 2nd or 4th chromosome. It should be also noted that Jagadeeswaran et al. (Jagadeeswaran et al., 2009) used a different sequence for miR399a than the one present in miRBase as the 20th nucleotide was an uracil instead of an adenine although this change does not affect the outcome of the blast. The reason for psRNATarget tool not giving the E2 ligase as a candidate target was that the library used does not have this probable E2 ligase, found in BLAST tool, registered. By inserting both sequences of predicted protein E2 ligases found by the BLAST tool, in the psRNATarget tool the outcome is similar to the one found by Jagadeeswaran et al. (Jagadeeswaran et al., 2009) as it predicted 5 places of cleavage in the 2nd chromosome protein with one having a worse score than the others. It should be noted that the results are only similar and not the same due to the 20th nucleotide being different and that these cleavage sites have a better score than those initially found with psRNATarget tool with just the library from Phytozome.

Plant, Pod and Seed phenotypes observed

Although the data gather for plant, pod and seed phenotypes was scarce, some important observations were made. First, there was the observation of fewer seeds per pod in Mtr-MIM408 plants, which is in accordance with what is seen in other plants as well as in *M.truncatula* when miR408 is downregulated (Trindade, 2012; Song et al., 2018; Zhang et al., 2018). This was expected as the downregulation of miR408 by a mimic would lead to the accumulation of PLC which in higher concentrations leads to lower pollen fertility rate so less seeds are expected. The second observation is the delayed blossoming of the flowers in Mtr-MIM408 plants and since in *A. thaliana* the overexpression of miR408 lead to an accelerated growth of the plants due to higher net photosynthetic rate (Song et al., 2018), a downregulation of miR408 probably lead to a decreased net photosynthetic rate which slowed down the growth of the Mtr-MIM408 plants by 2 weeks and thus explaining the late blooming.

Binary Vectors and Transforming plants

One of the goals of this project was to obtain binary vectors that could be used by *A. tumefaciens* to infect and introduce the modified IPS1 gene in Medicago to act as a mimic of the miRNA´s target. While the method used to mutate the IPS1 worked, when the recombination of the mutated fragment and PDoNR was attempted, the resulting plasmid when used to transform

E. coli strain DH5α, no colonies were found in PDoNR/166a, PDoNR/408 or PDoNR/399a. Even after reconfirming the selection medium (spectinomycin) there was still no colony found and since much time was spent due to the mistakes committed and as the ongoing pandemic limited the time that could be spent on the laboratory it was decided to abandon this task and focus on the analysis of the levels of expression of miR408. Nevertheless, in the future, when this task is resumed, it is likely that the Gateway system will be replaced, as the cells were competent with control transformations succeeding and the recombination of PDoNR IPS1/MIM408 previously done by Trindade also failed, so either the LR recombination reaction failed due to the pk7WG2.0 no longer be able to recombine or the Gateway system itself may have a poor performance for this procedure. If it is due to the first hypothesis, it can be solved by acquiring a new stock of pk7WG2.0 and test again. If is due to the latter, then perhaps other alternatives to the Gateway system such as TA cloning or restriction enzymes and ligation might be chosen to insert the mimic fragment into a binary vector.

Transcript expression analysis in different stages of development

Regarding the analysis of transcript expression, even though all steps were successfully executed, no conclusions can be made due to the control samples not being suitable for analysis, as either there was a contamination of the control samples or the plants used for control are transformed with MIM408.

In case it is a contamination, as all control samples showed presence of MIM408 in RT-qPCR (tables 6.5 and 6.6) and later confirmed by amplification (figures 3.5 to 3.9) it is likely that the samples were contaminated during the storage of the seeds and pods in the freezer. The reasons behind this conclusion are, the samples were grounded in three different times so it is unlikely that in all these three events there was a contamination, the stage 2 pods also showed amplification of MIM408 and from extraction to cDNA synthesis only stage 2 pod's control samples were used. Other factor to take into account is, the Ct values of MIM408 in both Mtr-GFP and Mtr-MIM408 plants are few and late Ct, most likely because they were further diluted when the samples from Mtr-GFP and Mtr-MIM408 plants were mixed, as the control should not have the mimic. Even though the fold changes between Mtr-GFP and Mtr-MIM408 cannot be calculated, as explained above, they were nevertheless calculated (supplementary table 9 and 10) in hope of finding more information regarding the origin of MIM408 in control samples. With this analysis again it seems to confirm that indeed the samples are contaminated as in stage 1 samples the fold changes of PLC show that the Mtr-MIM408 has roughly only between 40 to 65% of the amount of PLC of the control plant. In stage 5 this decreases to just 20 to 25%. According to the bibliography (Trindade, 2012), Mtr-MIM408, which has the MIM408, reduces the availability of miR408 by binding to it, preventing the miR408/RISC from cleaving PLC transcripts and as less transcripts are cleaved there should be more of these transcripts present in transformed plants, but in reality the opposite is true.

The second alternative is the control plant used is also transformed with MIM408 and according to the fold changes it should have a higher expression of MIM408 to explain the inferior values of PLC of Mtr-MIM408 plant relative to Mtr-GFP. This explanation seems unlikely as the Mtr-MIM408 plant according to Trindade (2012) contained 5 copies of the mutated IPS1 gene linked to a CaMV 35S promoter (CaMV35S) so a higher expression of MIM408 was to be expected, while only low expression was registered. Another reason that makes this unlikely, is the late blooming observed in the transformed plants probably caused by the excess of PLC proteins, that are known to negatively impact the pollen germination, suggesting that the Mtr-MIM408 plants were the only ones transformed with MIM408 or in the unlikely scenario, expressed more MIM408 than control plants.

Nevertheless, in both scenarios the impact of the mimic on the expression of miR408 and its target cannot be measured and the usage of the chosen reference genes may also be invalid, as there is no guarantee that the presence of the mimic does not influence the expression of PPRep and GAPDH3. The only solution to this problem is to redo the experiment starting by planting new control and transformed plants. Other step to avoid contamination should be the testing of all plants for the presence of MIM408 by extracting RNA from leaves. Even if no conclusions can be made regarding the transcript expression, this experiment still managed to prove that the engineered Two-tailed primer was functional (supplementary table 7).

Conclusion

Overall, this work was able to produce the fragments of MIM166a and MIM399a required for later transformation and design and test the primers required for the analysis of the transcripts in study. More importantly, similar to previous studies, the number of seeds per pod was less in transformed plants (Mtr-MIM408) than control ones. Even if not all the tasks proposed were successfully completed, important data was still produced. Nevertheless, this work was an important learning experience, and much was learned with the mistakes made. With additional work it will be possible to understand the role of these miRNAs in the development of the seeds in different stages of development.

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Supplementary Tables and Figures

Figure 6.1: Nucleotide sequence of the forward strand from the IPS1 fragment mutated for MIM166a. In yellow is the 21 nt that bind to its target miRNA with lower case letters representing nucleotides that differ from the pretend mutation and the 3 letters in between, not marked in yellow, represent the bulge.

TGATTTTATTTTGACTGATAGTGACCTGTTCGTTGCAACAAATTGATGAGCAATGCTTTTTTA
TAATGCCAACTTTGTACAAAAAAGCAGGCTGCATGCTCCCGGCCGCCATGGCGGCCGCGG
GAATTCGATTGTGGATCCAAGAAAAATGGCCATCCCCTAGCTAGGTGAAGAAGAATGAAAA
CCTCTAATTTATCTAGAGGTTATTCATCTTTTAGGGGATGGCCTAAATACAAAATGAAAACT
CTCTAGTTAAGTGGTTTTGTGTTCATGTAAGGAAAGCGTTTTAAGATATGGAGCAATGAAGA
CTGCAGAAGGCTGATTCAGACTGCGAGTTTTGTTTATCTCCCTCTAAAAAGGGGAATGAA**a**
*ATA*CCTGGTCC**a**AACCTTCGGTTCCCCTCGAAATCAGCAAATTATGTATCTTTAATTTTGTAA
TACTCTCTCTCTTCTCTATGCTTTGTTTTTCTTCATTATGTTGGGGTTGTACCCACTCCCGCG
CGTTGTGTGTTCTTTGTGTGAGGAATAAAAAAATATTCGGATTTGAAAACTAAAACTAGAGT
ATTTTTATTGATATTCTTGTTTTTCATTTA*TATCTAATAAGTTTGGAGAATAGTCAAACCAGT
GCATGTAAATTTGCTTCCAATTCTCTTTATAGTGAATTCCTCCCCGGGCAGAAACCCA*CTTT
CTTGTACAAAGTTGGCATTATAA*AAAGCATTGCTTATCATTTTGTTGCAACGAACAGGTCAC
TATCAGTCAAAATAAAA*TCTTTATTTGC-G-CCG-CT-ATA-CCCC-
TA**GTGAGTCGTATTA*TGTCATA-CTGTTTCC**G**ATA

Figure 6.2: Nucleotide sequence of the reverse strand from the IPS1 fragment mutated for MIM166a. In yellow is the 21 nt that bind to its target miRNA and the 3 letters in between, not marked in yellow, represent the bulge.

T***ATAGGGGATATA*CTGGATGGCAAATAATGATTTTATTTTGACTGATAGTGACCTGTTCG
TTGCAACAAATTGATAAGCAATGCTTTCTTATAATGCCAACTTTGTACAAGAAAGCTGGGTT
TCTGCCCGGGGAGGAATTCACTATAAAGAGAATCGGAAGCAAATTTACATGCACTGGTCTG
ACTATTCTCCAAACTTATTAGATACTAAATGAAAAACAAGAATATCAATAAAACTACTCTAGT
TTTAGTTCTCAAATCCGAATATTTTTTTATTCCTCACACAAAGAACACACAACGCGCGGGAG
TGGGTACAACCCAAACATAATGAAGAAAAACAAAGCATAGAGAAGAGAGAGAGTATTACAA
AATTAAAGATACATAATCTGCTGATTCCGAGGGGAACCGAAGCTTCGGACCAGGTATCTTC
ATTCCCCTTTCTAGAGGGAGATAAACAAAACTCGCAGTCTGAATCAGCCTTCTGCAGTCTT
CATTGCTCCATATCTTAAAACGCTTTCCTTACATGAACACAAAACCACTTAACTAGAGAGTTT
TCATTTTGTATTTAGGCCATCCCCTAAAAGATGAATAACCTTTAAATAAATTAGAGGTTTTCA
TTCTTCTTCACCTAGCTAGGGGATGGCCATTTTTCTTGGATCCACAATCGAATTCCCGCGG
CCGCCATGGCGGCCGGGAGCATGCAGCCTGCTTTTTTGTACAAAGTTGGCATTATAAAAAA
GCATTGCTCATCAATTTGTTGCAACGAACAGGTCACTATCAGTCAAAATAAAATCATTATTT
GGGGCCCGA*CT*AAAACTGGCCG*CGTTTTACCTCGAGCATTA

Figure 6.3: Nucleotide sequence of the forward strand from the IPS1 fragment mutated for MIM399a. In yellow is the 21 nt that bind to its target miRNA and the 3 letters in between, not marked in yellow, represent the bulge.

*******TGATTTTATTTT*ACTGATAGTGACCTGTTCGTTGCAACAAATTGATGAGCAATGCTTT
TTTATAATGCCAACTTTGTACAAAAAAGCAGGCTGCATGCTCCCGGCCGCCATGGCGGCC
GCGGGAATTCGATTGTGGATCCAAGAAAAATGGCCATCCCCTAGCTAGGTGAAGAAGAAT
GAAAACCTCTAATTTATCTAGAGGTTATTCATCTTTTAGGGGATGGCCTAAATACAAAATGA
AAACTCTCTAGTTAAGTGGTTTTGTGTTCATGTAAGGAAAGCGTTTTAAGATATGGAGCAAT
GAAGACTGCAGAAGGCTGATTCAGACTGCGAGTTTTGTTTATCTCCCTCTAAAAACTGGGC
AAATCCTATCCTTTGGCAAGCTTCGGTTCCCCTCGGAATCAGCAAATTATGTATCTTTAATT
TTGTAATACTCTCTCTCTTCTCTATGCTTTGTTTTTCTTCATTATGTTTGGGTTGTACCCACT
CCCCCGCGTTGTGTGTTCTTTGTGTGAGGAATAAAAAAATATTCGGATTTGAAAACTAAAAC
TAAAGTATTTTTATTGATATTCTTGTTTTTC*TTTA*TATCTAATAATTTTGGAGAATATTCAAA
CCAGTGCATGTAAATTTGCTTCC*ATTCTCTTTATA*TGAATTCCTCCCCGGGCAGAAACCCA
GCTTTCTTGTACAAAGTTGGCATTATAAAAAAGCATTGCTTATCAATTTGTTGCACGAACAG
GTCACTATCAGTCAAAAAAAATCATTATTTGC*GTC*GAGT*AACCCG*TGTG*****ATAACTGG
CCATA**TGTTT***G**

Figure 6.4: Nucleotide sequence of the reverse strand from the IPS1 fragment mutated for MIM399a. In yellow is the 21 nt that bind to its target miRNA and the 3 letters in between, not marked in yellow, represent the bulge.

****T***GGG**ATCAGCTGGATGGCA*TAATGATTTTATTTTGACTGATAGTGACCTGTTCGTT
GCAACAAATTGATAAGCAATGCTTTCTTATAATGCCAACTTTGTACAAGAAAGCTGGGTTTC
TGCCCGGGGAGGAATTCACTATAAAGAGAATCGGAAGCAAATTTACATGCACTGGTCTGAC
TATTCTCCAAACTTATTAGATACTAAATGAAAAACAAGAATATCAATAAAACTACTCTAGTTTT
AGTTCTCAAATCCGAATATTTTTTTATTCCTCACACAAAGAACACACAACGCGCGGGAGTGG
GTACAACCCAAACATAATGAAGAAAAACAAAGCATAGAGAAGAGAGAGAGTATTACAAAATT
AAAGATACATAATCTGCTGATTCCGAGGGGAACCGAAGCTTGCCAAAGGATAGGATTTGCC
CAGTTTCTAGAGGGAGATAAACAAAACTCGCAGTCTGAATCAGCCTTCTGCAGTCTTCATT
GCTCCATATCTTAAAACGCTTTCCTTACATGAACACAAAACCACTTAACTAGAGAGTTTTCAT
TTTGTATTTAGGCCATCCCCTAAAAGATGAATAACCTCTAGATAAATTAGAGGTTTTCATTCT
TCTTCACCTAGCTAGGGGATGGCCATTTTTCTTGG.

Figure 6.5: Nucleotide sequence of the forward strand from the IPS1 fragment mutated for MIM408. In yellow is the 21 nt that bind to its target miRNA and the 3 letters in between, not marked in yellow, represent the bulge. Unlike MIM166a and MIM399a, that were mutated during this work, this is a sequencing of the fragment MIM408 mutated by Trindade (Trindade, 2012).

**********TGATTT*ATTTTGACTGATAGTGACCTGTTCGTTGCAACAAATTGATGAGCAATGCT
TTTTTATAATGCCAACTTTGTACAAAAAAGCAGGCTGCATGCTCCCGGCCGCCATGGCGGC
CGCGGGAATTCGATTGTGGATCCAAGAAAAATGGCCATCCCCTAGCTAGGTGAAGAAGAA
TGAAAACCTCTAATTTATCTAGAGGTTATTCATCTTTTAGGGGATGGCCTAAATACAAAATG
AAAACTCTCTAGTTAAGTGGTTTTGTGTTCATGTAAGGAAAGCGTTTTAAGATATGGAGCAA
TGAAGACTGCAGAAGGCTGATTCAGACTGCGAGTTTTGTTTATCTCCCTCTAGAAAGCCAG
GGAAGGCTTGGCAGTGCATAGCTTCGGTTCCCCTCGGAATCAGCAGATTATGTATCTTTAA
TTTTGTAATACTCTCTCTCTTCTCTATGCTTTGTTTTTCTTCATTATGTTTGGGTTGTACCCAC
TCCCGCGCGTTGTGTGTTCTTTGTGTGAGGAATAAAAAAATATTCGGATTTGAGAACTAAAA
CTAGAGTAGTTTTATTGATATTCTTGTTTTTCATTTAGTATCTAATAAGTTTGGAGAATAGTCA
GACCAGTGCATGTAAATTTGCTTCCGATTCTCTTTATAGTGAATTCCTCCCCGGGCAGAAAC
CCAGCTTTCTTGTACAAAGTTGGCATTATAAGAAAGCATTGCTTATCAATTTGTTGCAACGA
ACAGGTCACTATCAGTCAAAATAAAATCATTATTTGCCATCCAGCTGATATCCCCTATAGTG
AGTCGTATTACATGGTCATAGCTGTTTCCTGGCAGCTCTGGCCCGTGTCTCAAAATCTCTG
ATGTTACATTGCACAAGATAAAAATATATCATCATGAACAATAAAACTGTCTGCTTACATAAA
CAGTAATACA*GGGGTGTTATGAGC**TATTCAACGGGAAACGTCGAGGCCGCGATTAAATT
CCAACATGGATGCTGATTTATATGGGTA*AAATGGGCT*CGCGATAATGTCGGGCAATC***G
CGA**T***ATCGCTTGTATGGGAAGCCGGATGCGCCA*A*TTGTTTTC*GAAAC*TGG**AAGG*
AGCGTTGCCAATGATG**C*GA*GA*ATGGTCA**ACTAAA*****GAC*G*ATTTAT**C*CT*TTCC
GAACA**A*GC**TT*ATCCG*****C*G***G*A**********C***C***G****C*********C**TTCAGG*AT*A
********CCC*G*A*******G*****A*******

Figure 6.6: Nucleotide sequence of the reverse strand from the IPS1 fragment mutated for MIM408. In yellow is the 21 nt that bind to its target miRNA and the 3 letters in between not marked in yellow represent the bulge. Unlike MIM166a and MIM399a, that were mutated during this work, this is a sequencing of the fragment MIM408 mutated by Trindade (Trindade, 2012).

CCTTAGGGGAATCAGCTGGATGGCAAATAATGATTTTATTTTGACTGATAGTGACCTGTTCG
TTGCAACAAATTGATAAGCAATGCTTTCTTATAATGCCAACTTTGTACAAGAAAGCTGGGTT
TCTGCCCGGGGAGGAATTCACTATAAAGAGAATCGGAAGCAAATTTACATGCACTGGTCTG
ACTATTCTCCAAACTTATTAGATACTAAATGAAAAACAAGAATATCAATAAAACTACTCTAGT
TTTAGTTCTCAAATCCGAATATTTTTTTATTCCTCACACAAAGAACACACAACGCGCGGGAG
TGGGTACAACCCAAACATAATGAAGAAAAACAAAGCATAGAGAAGAGAGAGAGTATTACAA
AATTAAAGATACATAATCTGCTGATTCCGAGGGGAACCGAAGCTATGCACTGCCAAGCCTT
CCCTGGCTTTCTAGAGGGAGATAAACAAAACTCGCAGTCTGAATCAGCCTTCTGCAGTCTT
CATTGCTCCATATCTTAAAACGCTTTCCTTACATGAACACAAAACCACTTAACTAGAGAGTTT
TCATTTTGTATTTAGGCCATCCCCTAAAAGATGAATAACCTCTAGATAAATTAGAGGTTTTCA
TTCTTCTTCACCTAGCTAGGGGATGGCCATTTTTCTTGGATCCACAATCGAATTCCCGCGG
CCGCCATGGCGGCCGGGAGCATGCAGCCTGCTTTTTTGTACAAAGTTGGCATTATAAAAAA
GCATTGCTCATCAATTTGTTGCAACGAACAGGTCACTATCAGTCAAAATAAAATCATTATTT
GGGGCCCGAGCTTAAGACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACATCCATG
CTAGCGTTAACGCGAGAGTAGGGAACTGCCAGGCATCAAATAAAACGAAAGGCTCAGTCG
GAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCCTGAGTAGGACAA
ATCCGCCGGGAGCGGATTTGAACGTTGTGAAGCACCGGCCCGAAGGGTGGTGGTTAGGA
GG

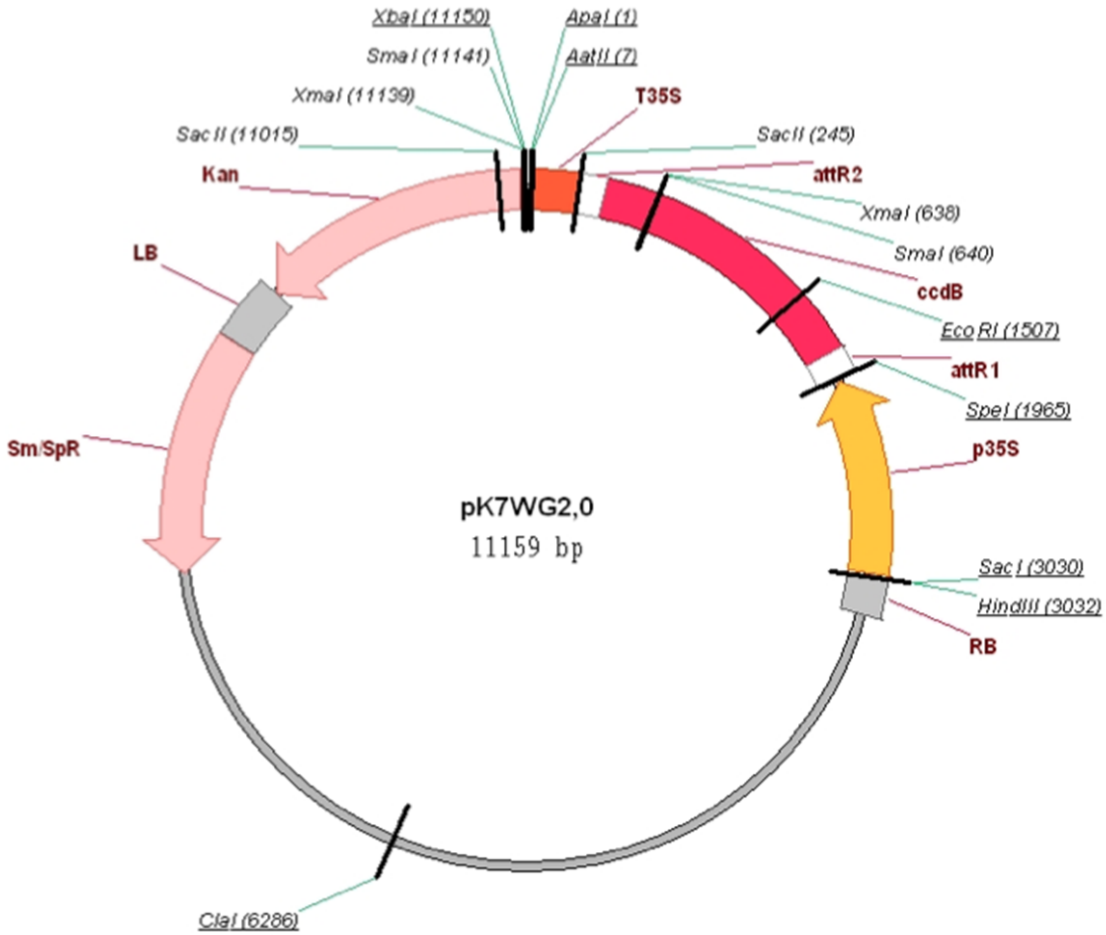

Figure 6.7: pK7WG2.0 binary vector Gateway® system for *A. tumefaciens* (Karimi et al., 2002).

Table 6.1: Ct values of the reference genes of the transformed plant for stage 1 of development

Concentration (ng)	**10**	**5**	**1**	**0.1**
Log_{10}	**1**	**0.69897**	**0**	**-1**
GAPDH3 1	30.62	32.00	34.01	
GAPDH3 2	22.17	23.51	25.69	29.04
Aprt 1	23.97	24.95	26.59	31.94
Aprt 2	23.6	25.56	30.22	33.23
Mtc 27 1	19.11	20.71	23.34	26.40
Mtc 27 2	19.88	20.75	22.55	25.84
Act2	25.31	25.63	28.08	31.76
L2	24.05	25.53	28.13	31.29
PPRep1	26.79	27.95	30.36	34.27
PPRep 2		27.59	29.97	34.11
PDF2 1	28.51	29.07	31.93	35.03
PDF2 2		29.56	33.03	36.58

Table 6.2: Ct values of the reference genes of the transformed plant for stage 1 of development

Concentration (ng)	**10**	**5**	**1**	**0.1**
Log_{10}	**1**	**0.69897**	**0**	**-1**
GAPDH3 1	22.09	23.74	25.71	29.045
GAPDH3 2	23.21	24.085	26.725	30.275
Aprt 1	24.78	26.31	26.19	29.84
Aprt 2	24.69	25.47	27.97	31.33
Mtc 27 1	23.01		23.38	26.8
Mtc 27 2	19.7	19.94		33.76
Act2 1	23.96	25.34	27.91	31.36
Act2 2	23.81	24.65	27.33	31.86
L2 1	22.51	26.57	26.32	30.03
L2 2	22.34	25.79	27.51	29.87
PPRep1	26.94	28.2	30.64	33.98
PPRep 2	26.12	26.93	29.86	32.6
PDF2 1	29.71	30.57	33.97	36.56
PDF2 2	26.89	27.77	30.19	33.98

Table 6.3: Ct values of the reference genes of the control plant for stage 5 of development

Concentration (ng)	**10**	**5**	**1**	**0.1**
Log_{10}	**1**	**0.69897**	**0**	**-1**
GAPDH3	24.57	25.77	28.06	31.68
Aprt	31.84	31.46	37.01	
Mtc 27	22.24	22.19	24.62	28.11
Act2 1	27.87	28.57	31.07	35.72
Act2 2	28.33	29.66	31.13	34.73
L2 1		28.47	34.1	37.74
L2 2		25.71	27.85	32.17
PPRep1	28.89	29.62	32.77	37.15
PPRep 2	29.79	30.1	32.72	37.14
PDF2 1	33.09	33.9	35.44	40
PDF2 2	32.99	33.76	36.28	

Table 6.4: Ct values of the reference genes of the control plant for stage 5 of development

Concentration (ng)	**10**	**5**	**1**	**0.1**
Log_{10}	**1**	**0.69897**	**0**	**-1**
GAPDH3 1	22.34	23.5	25.785	29.09
GAPDH3 2	22.37	23.225	25.38	29.16
Aprt 1	19.16	28.36	27.75	29.1
Aprt 2	19.71	20.39	23.31	26.11
Mtc 27 1	29.83	29.52	33.89	35.92
Mtc 27 2	28.6	29.93	32.86	36.71
Act2 1	23.64	25.3	27.17	32.11
Act2 2	24.77	25.5	29.69	40
L2 1	25.14	23.15	25.68	32.37
L2 2	23.25	25.57	26.35	29.35
PPRep1	26.83	27.69	29.86	33.16
PPRep 2	27.47	28.36	30.89	34.48
PDF2 1	28.34	29.09	33.21	36.66
PDF2 2	28.49	29.55	32.25	35.71

Table 6.5: Ct values of Plantacyanin (PLC), MIM408 and the genes chosen for reference in stage 1 pods. Trans- transformed plant; Ctl – control plant

Concentration (ng)	**10**	**5**	**1**	**0.1**
Log_{10}	1	0.69897	0	-1
Trans PLC 1	31.12	28.16	36.63	33.89
Trans PLC 2	27.08	27.97	30.24	37.39
Ctl PLC 1	31.03	33.35	30.59	34.03
Ctl PLC 2	26.85	26.88	30.37	33.93
Ctl PLC 3	25.25	26.98	29.05	34.66
Ctl PLC 4		27.07	32.3	33.03
Trans MIM 1	31.04	40		35.82
Trans MIM 2			38.27	34
Ctl MIM 1				
Ctl MIM 2				
Ctl MIM 3	31.79	33.64	34.38	34.65
Ctl MIM 4		32.86		36.94
Trans GAPDH3	22.65	22.92	25.21	28.53
Ctl GAPDH3 1	22.66	22.84	25.22	28.66
Ctl GAPDH3 2	23.02	24.75	27.2	30.22
Trans PPRep	27.57	27.28	29.69	34.45
Ctl PPRep 1	27.44	29.7	30.64	33.69
Ctl PPRep 2	27.61	28.61	31.52	34.12

Table 6.6: Ct values of Plantacyanin (PLC), MIM408 and the genes chosen for reference in stage 5 pods. Trans- transformed plant; Ctl – control plant

Concentration (ng)	**10**	**5**	**1**	**0.1**
Log_{10}	1	0.69897	0	-1
Trans PLC 1	28.75	29.34	32.22	35.21
Trans PLC 2	28.76	29.76	32.47	37.22
Ctl PLC 1	24.22	25.52	28.17	31.52
Ctl PLC 2	24.83	25.91	28.48	31.6
Ctl PLC 3	27.05	25.19	28.39	31.15
Ctl PLC 4	24.93	25.2	27.67	31.04
Trans MIM 1	35.4	34.97	37	
Trans MIM 2		34.67		
Ctl MIM 1			40	
Ctl MIM 2			37.13	
Ctl MIM 3				
Ctl MIM 4	36.19	40	35.9	
Trans GAPDH3	24.81	25.52	28.08	31.18
Ctl GAPDH3 1	22.27	23.32	25.99	29.2
Ctl GAPDH3 2	22.54	22.89	25.39	28.73
Trans PPRep	28.94	30.05	32.08	34.92
Ctl PPRep 1	26.68	27.8	30.19	33.16
Ctl PPRep 2	27.09	27.55	30.11	33.09

Table 6.7: Ct values of miR408 and the genes chosen for reference in stage 1 and 5 pods and Ct values of miR 408 of leaves from m9-10a embryogenic line. Trans- transformed plant; Ctl – control plant; Leaf - leaves from m9-10a embryogenic line.

Concentration (ng)	**10**	**5**	**1**	**0.1**
Log_{10}	**1**	**0.69897**	**0**	**-1**
Trans 408 s1 d1	31.15	33.16	35.61	40
Trans 408 s1 d2	32.05	33.56	35.82	
Ctl 408 s1 d1	29.11	30.44	32.56	35.68
Ctl 408 s1 d2	29.22	30.84	33.84	36.67
Trans 408 s5 a1	30.35	31.75	34.38	36.62
Trans 408 s5 a2	29.68	31.55	34.89	37.07
Ctl 408 s5 b1	29.9	31.11	34.78	37.3
Ctl 408 s5 b2	29.97	31.2	33.75	36.91
Leaf 408 1	24.87	26.56	29.5	32.54
Leaf 408 2	25.02	26.31	28.7	32.76
Trans GAPDH3 s1	21.19	23.86	24.16	32.04
Ctl GAPDH3 s1	22.87	25.01	26.49	29.97
Trans GAPDH3 s5	24.91	26.14	28.9	32.22
Ctl GAPDH3 s5	21.38	22.68	25.06	29.2
Trans PPRep s1	40	27.1	28.83	32.27
Ctl PPRep s1	28.37	27.59	30.15	35.37
Trans PPRep s5	38.56	30.01	32.53	
Ctl PPRep 1 s5	26.16	27.88	29.52	33.02

Figure 6.7: Integrity of the RNA extracted from *M.truncatula*. On the left is RNA extracted from control plants' pods and seeds and on the right from pods and seeds of transformed plants. Stage 1 of development (S1); Stage 5 of development (S5).

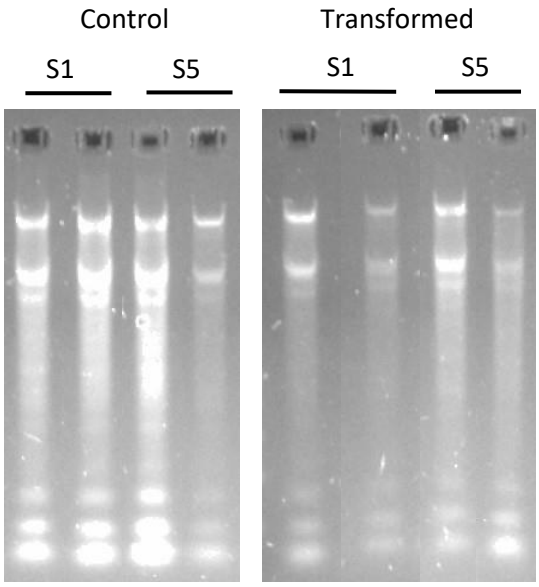

Figure 6.8: Integrity of the RNA extracted from M.truncatula. On the left is RNA extracted from control plants' pods from stage 2 of development, o the right is RNA extracted from leaves of M9-10a genotype.

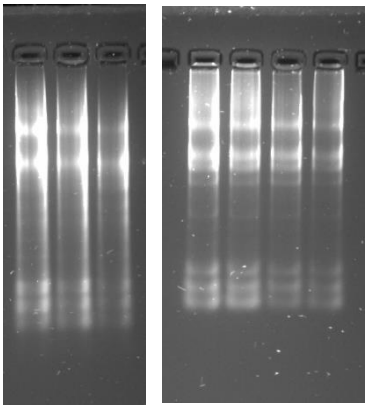

Table 6.8: RNA measured values in Nanodrop ND-2000C.

	Concentration (ng/µl)	**A260**	**A280**	**260/280**	**260/230**
Control S1 a	376.8	9.421	4.414	2.13	2.43
Control S1 b	350	8.750	4.132	2.12	1.99
Control S5 a	288.1	7.204	3.366	2.14	2.07
Control S5 b	315.3	7.882	3.670	2.15	2.23
Transformed S1 a	183.1	4.578	2.148	2.13	2.10
Transformed S1 b	120.4	3.010	1.541	1.95	1.67
Transformed S5 a	141.8	3545	1.665	2.13	2.05
Transformed S5 b	178.9	4473	2.095	2.14	2.15
Pod S2 1	736.9	18.422	8541	2.16	2.30
Pod S2 2	533.9	13.347	6.240	2.14	2.22
Pod S2 3	407.5	10.166	4.745	2.15	2.22
Leaf 1	528	13.200	6.180	2.14	2.24
Leaf 2	415	10.375	4.836	2.15	2.24
Leaf 3	281.1	7.028	3.294	2.13	2.28
Leaf 4	332.7	8.317	3.878	2.14	2.20

Table 6.8: Efficiency of the primers used in RT-qPCR.

Gene	Stage 1 (S1)	Stage 5 (S2)	Log10 (S1)	Log10 (S5)
Plantacynin	95.50%	95.50%	1.98	1.98
MIM408	ND*	ND*	ND*	ND*
Act2	93.33%	87.10%	1.97	1.94
GAPDH3	97.72%	95.50%	1.99	1.98
PDF2	93.33%	95.50%	1.97	1.98
PPRep	91.20%	93.33%	1.96	1.97
Mtc27	95.50%	95.50%	1.98	1.98
Aprt	87.10%	74.13%	1.94	1.87
L2	85.11%	81.28%	1.93	1.91
miR408	91.20%	93.33%	1.96	1.97

*ND: not determined due to no linearity between the Ct values of the dilutions.

Table 6.9: LB medium composition (Duchefa, Haarlem, NL).

Luria Broth Base, Miller	NaCl	0.5 g in 15.5 g of Luria Broth
	Tryptone	10 g in 15.5 g of Luria Broth
	Yeast extract	5 g in 15.5 g of Luria Broth
LB liquid medium	Luria Broth Base, Miller	15.5 g/L
	ddH_2O	
LB agar medium	Luria Broth Base, Miller	15.5 g/L
	agar	15 g/L
	ddH_2O	

Table 6.10: S.O.C medium composition (Takara Bio, Kusatsu, JP).

Bacto tryptone	2%
Bacto yeast extract	0.5%
NaCl	10 mM
KCl	2,5 mM
$MgSO_4$	10 mM
$MgCl_2$	10 mM
Glucose	20 mM